THE BIOCHEMICAL JOURNAL

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The *in vitro* Protection of Cholinesterases Against some Organophosphorus Inhibitors

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This work was carried out in 1948–9 and formed the subject of confidential reports to the Ministry of Supply. It was part of a search for simple compounds which would protect cholinesterases against inhibition by such compounds as Tabun (ethyl *NN*-dimethylphosphoramidocyanidate), Sarin (*iso*propyl *P*-methylphosphonofluoridate) and ethyl-Sarin (*iso*propyl *P*-ethylphosphonofluoridate). As with DFP (di*iso*propyl phosphorofluoridate: Mackworth & Webb, 1948) inhibition by Tabun (Augustinsson, 1951, 1953) and Sarin (unpublished work of A. Todrick & L. T. D. Williams) is irreversible, and it is reasonable to suppose that inhibition by ethyl-Sarin is also irreversible. The starting point of our experiments was the supposition that since nothing definite was known about the active centre of cholinesterases, the catalytic activity was probably associated with the amino acid side chains. A number of amino acids were examined for their ability to protect cholinesterase *in vitro*; only dopa (3:4-dihydroxyphenylalanine) was effective, but in high concentration, and it was found that protective ability was a property of catechol derivatives. It appears that Augustinsson (1952), independently and about the same time, discovered the reaction of catechols with Tabun, and published his results almost simultaneously with those of Jandorf, Wagner-Jauregg, O'Neill & Stolberg (1952) on the reaction of phenols with DFP. The present paper deals mainly with the properties of Sarin, but sufficient data are also given to make it clear that the three groups of workers have studied different facets of the same phenomenon.

A brief account of this work has been published (Berry, 1953).

THEORETICAL

One of us (A.T.) in earlier unpublished work on Sarin in collaboration with L. T. D. Williams considered the case of irreversible inhibition by a labile inhibitor. The gist of their treatment is as follows:

Let I be an inhibitor combining irreversibly with an enzyme E by means of a bimolecular reaction of rate constant k_4. Then

$$\frac{-\mathrm{d}(E)}{\mathrm{d}t}=k_4(E-x)\,(I-y). \qquad (1)$$

If the inhibitor is simultaneously destroyed by unspecified side reactions of apparent unimolecular rate k_0, then

$$\frac{-\mathrm{d}(I)}{\mathrm{d}t}=k_4(E-x)\,(I-y)+k_0(I-y). \qquad (2)$$

It is supposed that only the changes in enzyme activity can be measured. From the above equations

$$\frac{\mathrm{d}(I)}{\mathrm{d}(E)}=1+\frac{k_0}{k_4(E-x)}, \qquad (3)$$

and if the enzyme activity (E_∞) is measured at a time when no further inhibition can be detected, and the inhibitor is presumed to be all destroyed,

$$(I)=x_\infty+\frac{k_0}{k_4}\ln\frac{(E_0)}{(E_\infty)}, \qquad (4)$$

where x_∞ is the amount of enzyme inhibited and (E_0) the initial activity. It can be calculated from Berry's (1951) data that the concentration of serum enzyme used in the present studies did not exceed 0·5 μmM, whilst the concentrations of Sarin used were upwards of 10 μmM. Therefore the term x_∞ can be neglected, as Todrick & Williams (unpublished) had assumed.

If to such a system is added a substance P which reacts bimolecularly with I, rate constant k_p, and if P is in excess of I, equation 2 now becomes

$$\frac{-\mathrm{d}(I)}{\mathrm{d}t}=k_4(E-x)\,(I-y)+(k_0+k_p(P))\,(I-y), \qquad (2a)$$

and equation 4 becomes

$$(I)=\frac{k_0+k_p(P)}{k_4}\ln\frac{(E_0)}{(E_p)}, \qquad (4a)$$

where (E_p) is the activity in presence of P corresponding to (E_∞) in its absence. Combining equations (4) and (4a) to eliminate (I).

$$\frac{\log(E_p)/(E_\infty)}{(P)\log(E_0)/(E_p)}=\frac{k_p}{k_0}=\text{constant}. \qquad (5)$$

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The above treatment deals with enzyme activities. Since the only measure of this is the velocity of breakdown of substrate, the corresponding velocities may be used provided that the substrate concentration is the same in all assays.

EXPERIMENTAL

The enzyme preparations were horse serum or rat-brain dispersions diluted with bicarbonate–Ringer to give an output of about 100 μl. CO_2/30 min. in the Warburg apparatus at 38°. The gas phase was 5% CO_2 in N_2, and with 25 mM-$NaHCO_3$ the pH was 7·4.

The substrates were benzoylcholine (6 mM final concentration) and acetylcholine (18 mM) respectively. They were kept as stock solutions in distilled water.

For most experiments Sarin was used as a stock solution in dry *n*-propanol, the alcohol being added to the controls, but some experiments were carried out with freshly prepared solutions of this and the other inhibitors in distilled water.

The 'protection test' which, with appropriate modifications, formed the basis of the experiments, consisted in adding inhibitor solution to the mixture of enzyme and substance being tested. The pH was brought to 7·4 if necessary. This mixture, of total volume 2·5 ml., was put into the main compartment of the Warburg vessels, and 0·5 ml. substrate solution in the side bulb. At this stage the mixture contained 0·125 ml. *n*-propanol, 0·67M. The respirometers were gassed, and incubated in the thermostat at 38° for 3 hr. to allow of complete destruction of the inhibitor. The substrate was then tipped, and the residual enzyme activity determined.

The extent to which enzyme activity in the presence of the substance being tested approached that of the control was a measure of the protection conferred. Since some of the substances examined were themselves slightly inhibitory, 'percentage protection' is defined as

$$100 \times \frac{\text{(percentage inhibition by Sarin)} - \text{(percentage inhibition by Sarin and compound)}}{\text{(percentage inhibition by Sarin)} - \text{(percentage inhibition by compound)}}.$$

Solutions of the oxidation products of dopa were prepared using Ag_2O, by a method similar to that of Mason (1948). The substances were not isolated, nor were their spectra checked. They were assumed to have the structure assigned by Mason, viz. a red solution, 1:2-dihydroindole-2-carboxylic acid-5:6-quinone; a colourless solution, 5:6-dihydroxyindole-2-carboxylic acid; and a purple solution, indole-2-carboxylic acid-5:6-quinone.

RESULTS

The effect of various compounds on the inhibition of horse-serum cholinesterase by Sarin

Of sixteen representative amino acids, only dopa was found to be able to protect cholinesterase and it did not inhibit of itself. Trials of several phenols and of two aliphatic polyhydroxy compounds, propan-1:2-diol and D-glucose, indicated that protection could be conferred by any catechol derivative, and that blocking of the hydroxy groups diminished the protective potency. Table 1 shows the results obtained with 10 mM concentrations of the compounds tested.

In these tests the initial Sarin concentration was about 20 μmM, so that there was an enormous excess of the test substance in the mixtures. The effect of diluting some of the more active substances is shown in Table 2, which shows that the protective action is mostly absent at a concentration of only 0·1 mM. Almost identical results were obtained using rat-brain dispersions.

No evidence could be obtained to suggest that dopa could reverse the inhibition caused by Sarin.

The action of dopa on other inhibitors

Our initial failure to repeat our work with Sarin was traced to the fact that we used water, not propanol, as solvent. It will be seen from Table 3 that for the most part propanol appears to confer protection against the inhibitors, since in its presence the I_{50} (concentration of inhibitor giving 50% inhibition) is increased. This is probably

Table 1. *Effect of compounds related to* 3:4-*dihydroxyphenylalanine on the inhibition of horse serum cholinesterase by* iso*propyl methylphosphonofluoridate* (*Sarin*)

Percentage protection is defined in the text. Compounds tested at 10 mM.

Group 1. Compounds which inhibit so strongly as to mask any protective action:
1:2-Dihydroxynaphthalene, 2:3-dihydroxynaphthalene, *p*-benzoquinone.

Group 2. Compounds failing to protect:
Glycine, alanine, serine, threonine, lysine, arginine, histidine, tryptophan, hydroxyproline, glutamic acid, aspartic acid, cysteine, methionine, phenylalanine, tyrosine, propan-1:2-diol, D-glucose, *m*-aminophenol, 3:4-dimethoxyphenylalanine, adrenochrome.

Group 3. Protective action 15–30%:
Phenol, *m*-dihydroxybenzene, *o*-aminophenol, *p*-aminophenol, *o*-dihydroxybenzene monomethyl ether, *o*-dihydroxybenzene dimethyl ether, 3-methoxy-4-hydroxyphenylalanine.

Group 4. More than 70% protection:
p-Dihydroxybenzene, *o*-dihydroxybenzene, L- and DL-3:4-dihydroxyphenylalanine, β-(3:4-dihydroxyphenyl)-α-(tetrahydro-1′:4′-thiazin-4′-yl)propionic acid 1′:1′-dioxide, 2(3′:4′-dihydroxyphenyl)2-hydroxyethylmethylamine (adrenaline), 1:2:4-trihydroxybenzene.

associated with the finding that alcohols are competitive reversible inhibitors (Todrick, Fellowes & Rutland, 1951), and are able to block the approach of the organophosphorus compounds to the enzyme.

Table 4 shows that on increasing the concentration of Sarin, the protective effect of 10 mM dopa diminishes, and that the protection is more in the presence of propanol, in spite of the greater concentration of Sarin used.

Table 2. *Effect of various concentrations of some phenols on the inhibition of horse-serum cholinesterase by Sarin*

	Percentage protection at concentration of phenol of		
	10 mM	1 mM	0·1 mM
DL-dopa	100	60	0
L-dopa	100	69	0
L-Adrenaline	81	52	0
Catechol	84	63	0
Pyrogallol	100	66	18
Hydroquinone	106	36	0

Table 3. *Effect of inhibitor diluent on inhibition of serum and brain cholinesterase*

The I_{50} is the concentration of inhibitor giving 50% inhibition.

	$I_{50} \times 10^9$ M			
	Serum cholinesterase		Brain cholinesterase	
Inhibitor	Water	*n*-Propanol (0·67 M)	Water	*n*-Propanol (0·67 M)
Tabun	11	6·5	—	—
Sarin	4	21	4	6·3
Ethyl-Sarin	2	4	—	—
DFP	3	8·2	80	240

Table 4. *Effect of inhibitor diluent on the protection by dopa against inhibition of serum cholinesterase by Sarin*

Diluent	Sarin (M × 10^9)	Percentage inhibition in absence of dopa	Percentage protection by 10 mM dopa
Water	5·0	56	52
	8·6	89	52
	54	95	41
	58	96	31
n-Propanol (0·67 M)	23	56	74
	36	88	98
	50	90	81
	54	86	86
	86	99	76
	180	99	50

Tables 5 and 6 indicate that the protection against other inhibitors is quantitatively similar, and that propanol tends to improve the protection.

Studies on the mechanism of protection

Among the possibilities examined were, that a direct dopa–Sarin (or other inhibitor) reaction occurs, and that dopa was the precursor of a much more active molecule. Evidence in favour of the first possibility is given here.

Quantitative studies on the dopa–Sarin interaction. The protection test was modified so that the time allowed for a possible dopa–Sarin reaction could be varied from 0 to 40 min. at room temperature, before adding cholinesterase. The results are

Table 5. *Effect of dopa and of inhibitor diluent on inhibition of serum cholinesterase by organophosphorus compounds*

Inhibitor	Diluent	$I_{50} \times 10^9$ M	Percentage inhibition in absence of dopa	Percentage protection by 10 mM dopa
Tabun	Water	12	42	33
		18	63	22
	n-Propanol (0·67 M)	13	67	81
Sarin	Water	5	56	52
	n-Propanol (0·67 M)	23	56	75
Ethyl-Sarin	Water	2·5	59	42
	n-Propanol (0·67 M)	5	59	83
DFP	Water	6·7	84	15
	n-Propanol (0·67 M)	11	59	37

Table 6. *Effect of dopa and of inhibitor diluent on inhibition of brain cholinesterase by organophosphorus compounds*

Inhibitor	Diluent	$I_{50} \times 10^9$ M	Percentage inhibition in absence of dopa	Percentage protection by 10 mM dopa
Tabun	Water	3·9	75	68
	n-Propanol (0·67 M)	9·7	72	89
Sarin	Water	3·2	37	72
	n-Propanol (0·67 M)	7·9	56	86
DFP	Water	160	86	34
	n-Propanol (0·67 M)	330	75	43

Table 7. *Effect on protection of cholinesterase of varying the time of contact between dopa and Sarin before adding cholinesterase*

Cholinesterase activities are given as $v = \mu$l. CO_2/30 min. The regression coefficients in column 4 are the rates of increase of v/min. of contact. Dopa concentration 0·7 mM. Initial Sarin concentration about 15 μmM.

Conditions	No. of determinations	Mean activity at zero time	Regression coefficient and S.D.	P that regression coefficients are zero or identical
dopa + Sarin + cholinesterase	25	69·3	0·470 ± 0·105	0·001 } 0·01
Sarin + cholinesterase	15	39·3	0·098 ± 0·05	0·05–0·1 }
dopa + n-propanol (0·67 M) + cholinesterase	10	100·2 ± 4·91	(100·1 ± 6·51 at 40 min.)	—
Cholinesterase + n-propanol (0·67 M)	8	104·2 ± 1·5	—	—

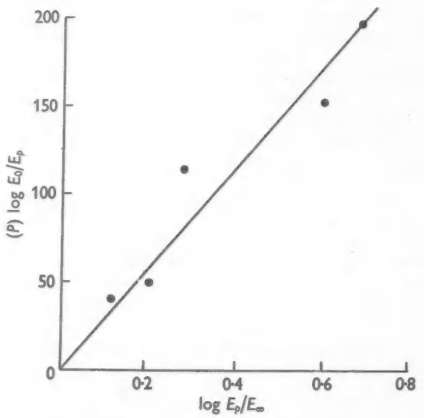

Fig. 1. Test of the existence of a direct reaction between dopa and Sarin. For explanation see text p. 4.

expressed as the increase of observed enzyme activity with time of contact of Sarin with the reaction mixture before adding cholinesterase. As Table 7 shows, the activity of the controls was unaltered. The activity in the presence of Sarin only slowly increases, since the inhibitor is labile; but in the presence of dopa the increase is significantly greater, suggesting a progressive reaction between Sarin and dopa.

The results of experiments using Sarin concentrations between 10·5 and 35 μmM and dopa between 0·1 and 5 mM, were applied to equation 5, above, as shown in Fig. 1. The experimental points were grouped around a straight line, indicating agreement with the hypothesis.

Protective effect of dopa oxidation products. Among the work carried out in an attempt to explain why protection could be observed only in the presence of relatively enormous concentrations of catechols, were some experiments designed to find out if traces of oxidation products, which might conceivably have arisen during the preliminary aerobic manipulations at slightly alkaline pH, were more active than dopa. Since none of them, whether with the coloured solutions described above, or involving the use of a polyphenol oxidase preparation, yielded evidence suggesting the existence of a more active compound, these experiments will not be described in detail.

DISCUSSION

Our work was exploratory in character; and points about which our evidence was scanty or largely speculative have since been covered more fully by the work of others, notably Jandorf *et al.* (1952), who have provided the best possible existence of a reaction between catechol and di*iso*propyl phosphorochloridate, which is more reactive than DFP, by isolating the product. Their manometric studies of the reaction rates between DFP and various phenols have been paralleled by studies on Tabun by Augustinsson (1952). Our evidence of the relative protective activities of phenolic compounds against Sarin conforms broadly to the pattern shown by these studies, and our demonstration that dopa can protect against two other related inhibitors, suggests that this reaction between phenols and organophosphorus compounds may be a general one, although there is insufficient evidence as to the nature of the reactive group in organophosphorus compounds.

At one time we thought, on evidence which was largely speculative, that a partial explanation of the requirements for a very large excess of catechol was that an ion was involved. Attempts to produce a more active compound which had a larger ionic dissociation constant, e.g. nitrophenols, were unsuccessful because of the much greater inhibition of cholinesterase by the catechol which then resulted. Jandorf *et al.* (1952) have produced satisfactory evidence that it is the catecholate ion which reacts with DFP; it is reasonable, therefore, in view of the similarity of the *in vitro* enzyme studies, to assume that catecholate reacts with the other compounds studied.

Augustinsson (1952) seems to place some importance on the observation that adrenaline and noradrenaline can protect cholinesterase. However, our quantitative data show (Table 2) that adrenaline

cannot protect at or below 0·1 mM, and a single observation on adrenochrome, kindly given us by Dr Harley-Mason, shows that it is without protective effect. It is well known to pharmacologists that the concentration of adrenaline in tissues is seldom above 1 μg./g. (about 5 μM) and is often nearer 1 μg./kg. (5 μmM). Thus it seems extremely unlikely that mobilization of adrenaline could influence the inhibition of cholinesterase *in vivo*.

SUMMARY

1. The effect of sixteen amino acids on the inhibition of horse-serum cholinesterase by Sarin (*iso*propyl *P*-methylphosphonofluoridate) was examined. Only dopa (3:4-dihydroxyphenylalanine) was able, in high concentration, to abolish the inhibition *in vitro*.

2. It was found that catechol derivatives generally are able to protect cholinesterases *in vitro*. Both horse serum and rat brain cholinesterases are protected against Sarin and also against DFP (di*iso*propyl phosphorofluoridate), Tabun (ethyl-*NN*-dimethylphosphoramidocyanidate) and *P*-ethyl-Sarin (*iso*propyl *P*-ethylphosphonofluoridate).

3. Evidence is adduced to suggest that the basis of the protection mechanism is a reaction between catechol and inhibitor.

4. None of the oxidation products of dopa were found to have a greater activity than dopa itself.

We are indebted to the Chief Scientist, Ministry of Supply, for permission to publish and to Mr D. R. Davies for constant advice and encouragement.

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The Role of Glycerylphosphorylcholine and Glycerylphosphorylethanolamine in Liver Phospholipid Metabolism

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The recent demonstration of the presence of free glycerylphosphorylcholine (Schmidt, Hecht, Fallot, Greenbaum & Thannhauser, 1952) and glycerylphosphorylethanolamine (Campbell & Work, 1952; Walker, 1952; Ansell & Norman, 1953) in aqueous extracts of animal tissues has suggested that these compounds may be intermediates in the metabolism of phosphatidylcholine and phosphatidylethanolamine. The immediate phosphorus-containing precursors of these two phospholipids during their synthesis in tissues are still unknown and it is important to know whether glycerylphosphorylcholine (GPC) or glycerylphosphorylethanolamine (GPE) could fulfil this role by reacting with activated fatty acids (Schmidt, Hershman & Thannhauser, 1945). Kornberg & Pricer (1953) have recently shown that an enzyme obtained from liver can cause two coenzyme A-activated fatty acids to combine with glycerophosphoric acid, producing a phosphatidic acid.

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Experiments have therefore been made to measure the specific radioactivities of GPC and GPE in rat liver at various times after the injection of labelled phosphate. At the same time the specific radioactivities of liver phosphatidylethanolamine and phosphatidylcholine were measured using a recently devised method of investigating phospholipid labelling (Dawson, 1954*a*). A comparison of these isotopic results suggests that in liver neither GPC nor GPE can act as major phosphorus-containing precursors of the phospholipids. On the other hand, the specific radioactivity of phosphatidylcholine is consistent with the assumption that it is the precursor of GPC, and similarly phosphatidylethanolamine fulfils the criteria for being the precursor of GPE. This would suggest that in liver *in vivo* these water-soluble phosphate esters are present solely as intermediates in the catabolism of the liver phospholipids.

A preliminary report of these results has already been presented to the Biochemical Society (Dawson, 1954*b*).

EXPERIMENTAL

Female albino rats (175–188 g.) were used throughout and fed *ad lib.* a diet of rat cake containing 27% protein, 4·4% fat and 59% carbohydrate. Each rat was lightly anaesthetized with ether to facilitate the quantitative intraperitoneal injection of carrier-free labelled orthophosphate (approx. 2×10^6 counts/min./100 g. body wt.). At the end of the period allowed for ^{32}P-exchange the rat was deeply anaesthetized by the injection of thiopentone; the liver was quickly removed, rapidly blotted to remove surplus blood and frozen in liquid oxygen. Experiments were performed over a period of about 4 months, as it took 4–5 days to obtain results from one animal.

Separation of GPC and GPE

The method used for preparing an extract of the water-soluble phosphorus-containing compounds in liver was devised to minimize the possibility that GPC or GPE would be liberated from tissue phospholipids during the process. The pH was not allowed to depart from near neutrality, as there is evidence that these phosphate esters can be liberated from the tissue phospholipids under acid and alkaline conditions (Ansell & Norman, 1953; Dawson, 1954*a*).

The frozen sample of liver (1 g.) was crushed to a fine powder and added to a centrifuge tube containing 4 ml. of boiling water. The mixture was heated for 90 sec. in a boiling-water bath, and then treated with 6 ml. of ethanol, any aggregates of tissue being dispersed with a glass rod. After centrifuging, the turbid supernatant was shaken for 5 min. with 40 ml. of $CHCl_3$. The milky upper-layer was centrifuged hard for 15 min. and the aqueous phase, which was usually very slightly turbid, was passed through a column of Amberlite IR 100 (H^+ form) resin (8 cm. × 0·8 cm.^{2}). This removed metallic cations which, if left in the solution, would interfere with the subsequent paper chromatography of the water-soluble phosphorus compounds. The solution was shaken with 2 vol. of *iso*-butanol, which cleared it of any residual turbidity. The aqueous phase was then filtered, adjusted to pH 7·5 with NH_3 and concentrated *in vacuo* below 50°. It was then applied to a washed filter-paper sheet and a two-dimensional chromatogram developed using phenol–NH_3 and propanol–NH_3 solvents.

Extraction and alkaline hydrolysis of liver lipids

The precipitate obtained after the liver had been boiled in water and treated with ethanol, as described in the previous section, was suspended in the $CHCl_3$ which had been used to extract the ethanol from the supernatant. Ethanol (10 ml.) was added to the suspension, which was then brought to the boil. It was slowly cooled to 0°, filtered through glass wool, and the lipid solution shaken with 80 ml. of ice-cold 0·1 N-HCl. The milky lower-layer was centrifuged to break the emulsion and the slightly turbid $CHCl_3$ extract taken to dryness *in vacuo*. Half of these phospholipids were then mildly hydrolysed with alkali (0·2 M methanolic NaOH, 15 min. at 37°), and the hydrolysis products resolved by two-dimensional filter-paper chromatography according to a procedure previously described (Dawson, 1954*a*).

Determination of the specific radioactivities of P-containing spots on the chromatograms

The methods used for detecting P-containing spots on the chromatograms and assaying these spots for phosphorus and radioactivity have already been described (Ansell & Dawson, 1951; Dawson, 1954*a*). The presence of molybdate on the chromatogram seemed to catalyse the oxidation of the paper spots with perchloric acid. The procedure finally adopted for the oxidation was to place the paper spots in boiling tubes together with 2 ml. of 72% perchloric acid A.R. The tubes were then suitably shielded in order to prevent personal injury in the event of an explosion, and heated on an electrical Kjeldahl digestion heater. After 5–10 min. the mixture usually became colourless, but if necessary a few drops of hydrogen peroxide were added to complete the digestion. The contents were then evaporated to approximately 0·6 ml. A water-insoluble residue separated during the process, and generally adhered to the walls of the tube on adding water to dissolve the inorganic P, but when necessary the solution was filtered clear of suspended matter. The deposit did not affect the recovery of phosphorus from chromatographic spots.

Chromatography

Whatman no. 1 paper sheets were well washed in 2 N acetic acid before use. Sheets treated with ethylenediaminetetra-acetic acid generally gave inferior chromatograms. The composition of the chromatographic solvents used has been reported previously (Dawson, 1954*a*). Descending chromatography was used for the phenol–NH_3 solvent and ascending for the other solvents.

RESULTS

Two-dimensional chromatograms (phenol–NH_3; propanol–NH_3) of the water-soluble phosphorus-containing substances present in rat liver showed the GPE and GPC spots to be well separated from the other water-soluble phosphorus compounds present in the tissue. When a chromatogram was sprayed with ninhydrin before the acid-molybdate, there was exact coincidence of the ninhydrin-reacting spot and the phosphorus-containing spot at the GPE position, with R_F in phenol–NH_3 of 0·62 and in propanol–NH_3 of 0·42. The GPE spot was also inseparable from a synthetic L-α-GPE marker kindly supplied by Professor E. Baer, Toronto, Canada. The GPC spot on the chromatogram, with R_F in phenol–NH_3 of 0·88 and in propanol–NH_3 of 0·41, was also inseparable from an authentic sample of L-α-GPC prepared from ox pancreas by the method of Schmidt *et al.* (1945). When the GPC spot was cut out and eluted with boiling water it was possible to detect choline in the solution by precipitating it as its characteristic reineckate. The choline had presumably been formed by hydrolysis of the GPC after the chromatogram had been sprayed with acid-molybdate to detect the phosphorus-containing spots. No choline could be detected in the area of paper immediately surrounding the GPC spot.

In further experiments both GPC and GPE were chromatographically detected in similarly prepared extracts of rat brain, but here the water-soluble phosphorus-containing compounds were passed through a column of Amberlite IRA 400 (HCO_3^- form) before they were applied to paper chromatograms. The presence of free GPC in brain has not been previously reported. Its identity was confirmed by isolating choline from the chromatographic spot.

The recoveries of phosphorus from the GPC and GPE chromatographic spots enabled the minimum concentrations of these substances in liver to be calculated. The recovery of liver GPC showed a wide variation between animals and gave a mean for twelve rats of 6·2 mg./100 g. wet wt. (range 2·1–22·9). The corresponding mean value for liver GPE was 4·5 mg./100 g. wet wt. with individual recoveries varying between 1·8 and 9·0.

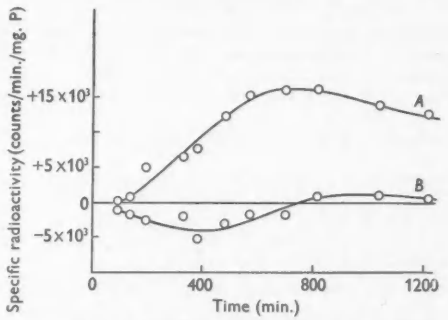

Fig. 1. *A*: curve showing the specific radioactivities of liver GPC at various times after labelled-phosphate injection. *B*: curve showing the specific radioactivities of liver GPC minus those of liver phosphatidylcholine at various times after labelled-phosphate injection. The points at a particular time represent the results obtained from one rat.

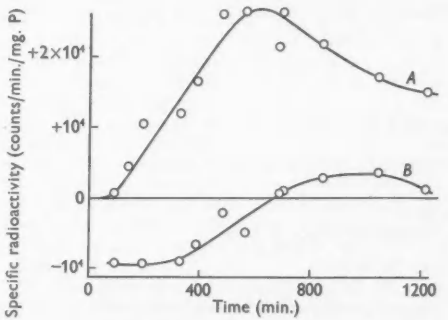

Fig. 2. *A*: curve showing the specific radioactivities of liver GPE at various times after labelled-phosphate injection. *B*: curves showing the specific radioactivities of liver GPE minus those of liver phosphatidylethanolamine at various times after labelled-phosphate injection. The points at a particular time represent the results obtained from one rat.

The specific radioactivities of the liver GPC and GPE at various times after the injection of labelled phosphate are illustrated in Figs. 1 and 2. A comparison is also made in these diagrams of the differences between the specific radioactivities of these substances and those of liver phosphatidylcholine and phosphatidylethanolamine. It can be seen (Fig. 1, curve *B*) that initially the liver phosphatidylcholine has a higher specific radioactivity than that of the GPC which, using the mathematically deduced criteria of Zilversmit, Entenman & Fishler (1943) would exclude GPC from the pathway by which the phospholipid becomes labelled with ^{32}P. On the other hand, the phospholipid appears to obey the correct radioactive criteria for being the precursor of GPC, i.e. initially the specific radioactivity of the phosphatidylcholine is above that of the GPC but its level then falls so that it equals that of the GPC when the latter reaches its maximum specific radioactivity. Subsequently the specific radioactivity of the phospholipid falls below that of the GPC. An essentially similar relationship is seen between the specific radioactivities of phosphatidylethanolamine and GPE (Fig. 2, curve *B*). Again GPE cannot act to any appreciable extent as an isotopic precursor of liver phosphatidylethanolamine, but instead the specific radioactivity/time curves suggest that the phospholipid is the precursor of the GPE.

DISCUSSION

By using certain assumptions Zilversmit *et al.* (1943) were able to deduce mathematically the relationship between the specific radioactivity of a precursor and its immediate metabolic product after the injection of a single dose of isotope. They predicted that the specific radioactivity of the precursor and product should be equal when the latter has reached its maximum specific radioactivity. Before this time the specific radioactivity of the precursor should be higher than the product, and afterwards lower than that of the product.

Although these predictions of Zilversmit *et al.* (1943) cannot be applied too rigorously to biological systems (Reiner, 1953), it can be seen that the present experiments provide no evidence that GPC or GPE can act *in vivo* as the isotopic precursors of the labelled liver phospholipids. If glycerophosphoric acid is a precursor of liver phospholipids (Zilversmit, Entenman & Chaikoff, 1948; Popjàk & Muir, 1950), the present results would suggest that fatty acids combine with it before the addition of a base. Recent work with isolated preparations has

indicated that enzyme systems exist in animal tissues which are capable of synthesizing phospholipids by such a process (Kornberg & Pricer, 1953; Kennedy, 1954; Dawson, 1954*a*). Ansell & Norman (1953) found that rat-brain GPE had a higher specific radioactivity than phosphatidylethanolamine at various times between 66 and 1455 min. after the injection of labelled phosphate. They concluded, however, that the magnitude of this difference was too small to allow GPE to act as a major precursor of cerebral phosphatidylethanolamine.

The relationships between the specific radioactivities of GPC and phosphatidylcholine and GPE and phosphatidylethanolamine indicate that these water-soluble phosphate esters exist in liver tissue solely as intermediaries in the catabolism of the phospholipids. An enzyme system capable of removing two fatty acids from phosphatidylcholine and phosphatidylethanolamine has not so far been reported in liver. Lecithinase *A* which removes a single unsaturated fatty acid has been found in rabbit liver (Francioli, 1934). Contardi & Ercoli (1935) obtained evidence that a compound which was probably GPC was formed on prolonged incubation of rice-bran extracts with lysolecithin. Schmidt *et al.* (1945) isolated GPC from autolysing ox pancreas, while Hayaishi & Kornberg (1954) recently described the accumulation of GPC when heat-treated extracts of *Serratia plymuthicum* were incubated with phosphatidylcholine.

SUMMARY

1. The specific radioactivities of the free glycerylphosphorylcholine and glycerylphosphorylethanolamine in rat liver have been measured by isolating them chromatographically at various times after the injection of labelled phosphate.

2. A comparison of their specific radioactivities with those of liver phosphatidylethanolamine and phosphatidylcholine has suggested that they cannot act as major isotopic precursors of these phospholipids.

3. Liver phosphatidylcholine obeys the correct radioactive criteria for being the precursor of glycerylphosphorylcholine, while similarly liver phosphatidylethanolamine fulfils the isotopic conditions necessary for being the precursor of glycerylphosphorylethanolamine.

The author is indebted to Sir Rudolph Peters, F.R.S., for his valuable advice.

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The Sulphatase of Ox Liver

4. A NOTE ON THE INHIBITION OF SULPHATASES *A* AND *B*

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The literature contains many conflicting reports on the influence of electrolytes on the activity of sulphatases, the early work being particularly difficult to interpret, as in many cases clear distinctions were not drawn between the enzymes obtained from different sources, such as mammalian liver, molluscan viscera, and *Aspergillus oryzae*. Recent work on mammalian liver sulphatase has also been unsatisfactory, as in all cases except that of previous work from this laboratory the enzymes used were unpurified preparations containing a number of different sulphatases, or even homogenates of tissues which had been treated with histological fixatives (Seligman, Chauncey & Nachlas, 1951). The present situation is extremely confused: sodium chloride has been reported to inhibit sul-

phatase in tissue sections, but to be without action on homogenates (Rutenburg, Cohen & Seligman, 1952) yet potassium chloride has been claimed (Dodgson, Spencer & Thomas, 1954*a*) to activate the sulphatase in homogenates. Magnesium chloride has similarly been claimed both as an inhibitor (Hommerberg, 1931) and an activator (Seligman *et al.* 1951). It has previously been shown that both sulphatases *A* and *B* are inhibited by sulphate ions (Roy, 1953*b*, 1954*a*), yet both Seligman *et al.* (1951) and Dodgson *et al.* (1954*a*) report the activation of crude sulphatase preparations by sulphate ions. This effect would appear very improbable on theoretical grounds alone.

The present paper describes an investigation of the effect of electrolytes on both crude ox-liver sulphatase and partly purified preparations of sulphatases *A* and *B*. Various other possible enzyme inhibitors have also been studied, and the results are reported below.

EXPERIMENTAL

The general experimental methods have already been described (Roy, 1953*a*, *b*, 1954*a*), the enzymic activity being followed by the colorimetric estimation of the 4-nitrocatechol liberated on hydrolysis of dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate). Sulphatases *A* and *B* were prepared as before, and in the present investigation were purified to stages *A*-3 and *B*-2 respectively.

The 5% (w/v) dispersion of ox liver in water used as the source of unfractionated enzyme was prepared by treating the tissue for 30 sec. in an Atomix blender at room temperature. The liver was used as soon as possible (usually about 1 hr.) after the slaughter of the animal.

Both in the case of the unfractionated enzyme and of sulphatases *A* and *B* an enzyme concentration was chosen such that approximately 100 μg. nitrocatechol were liberated under the experimental conditions described below.

Sulphatase *A* activity was estimated at pH 5·0 and a substrate concentration of 0·003 M nitrocatechol sulphate, and sulphatase *B* at pH 5·9 and 0·06 M nitrocatechol sulphate. The buffers used were sodium acetate, final concentration 0·15 M. The electrolytes were dissolved in the appropriate buffer and mixed with the substrate before the addition of the enzyme, so that the enzyme was treated simultaneously with the substrate and the electrolyte under investigation. Routine adjustments of pH were made with N acetic acid, not HCl as in previous work, since chloride ions have been found to have an influence on the activity of sulphatase *B*.

Since some of the other types of inhibitor were known to react only slowly with proteins, the buffered enzyme (0·25 M acetate, pH 5·0 and 5·9 for sulphatases *A* and *B* respectively) was treated with the appropriate concentration of inhibitor for 1 hr. at room temperature; then the inhibitor–enzyme solution was mixed with the appropriate substrate solution. Attempts to reverse the inhibition were carried out by adding the appropriate reagent to the enzyme–inhibitor mixture 10 min. before adding the substrate.

RESULTS

Influence of electrolytes

Unfractionated enzyme. Fig. 1 shows the effect of 0·025 M sodium sulphate and 0·08 M potassium chloride on the sulphatase activity of a 5% (w/v) dispersion of ox liver. In confirmation of the results of Dodgson *et al.* (1954*a*) it is obvious that at certain pH values both these compounds considerably enhanced the sulphatase activity of such a preparation. At other pH values an inhibition was noted in both cases. This effect was not shown by 0·001 M sodium sulphite, which inhibited strongly at all pH values. As these results were at variance with those previously reported (Roy, 1953*b*, 1954*a*) an investigation of the action of electrolytes on the activity of sulphatases *A* and *B* was undertaken.

Dodgson, Spencer & Thomas (1953), in their study of unfractionated rat-liver sulphatase, reported that the use of 0·2 M phosphate buffers in place of 0·5 M acetate buffers caused a shift in the pH optimum for the hydrolysis of *p*-acetylphenyl sulphate from pH 7·0 to 7·75. When phosphate buffers were used in the present study of the unfractionated ox enzymes no activity could be detected between pH 6·5 and 8·0. This discrepancy is resolved by the fact that the assay method used by Dodgson *et al.* (1953) estimates only the insoluble or microsomal enzyme (Dodgson, Spencer & Thomas, 1954*b*) which is not inhibited by phosphate (Dodgson & Spencer, private communication) and does not estimate the soluble enzymes derived from mitochondria (Dodgson *et al.* 1954*b*) which are inhibited by phosphate and which are essentially the enzymes assayed in the methods used in this

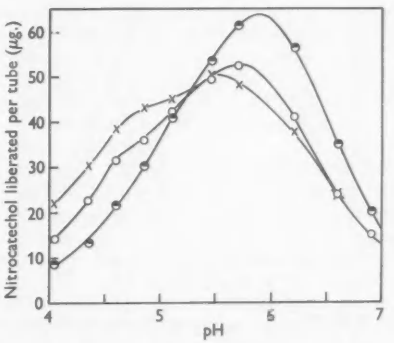

Fig. 1. Effect of electrolytes on the sulphatase activity of an untreated dispersion of ox liver. Assays in 0·15 M acetate buffers, substrate concentration 0·03 M nitrocatechol sulphate, total volume of reaction mixture 0·8 ml. containing 0·2 ml. enzyme solution. Incubated 1 hr. at 37°. ○—○, control; ●—●, 0·025 M-Na_2SO_4; ×—×, 0·08 M-KCl.

laboratory (Dodgson *et al.* 1954*b*; Roy, 1954*b*). Although this microsomal enzyme occurs in ox liver (Dodgson *et al.* 1954*b*) the amounts present would be too small to be detected by the present method so that no activity would be expected in the presence of phosphate. It is now proposed to refer to this microsomal sulphatase as sulphatase *C* until it is possible to name the various sulphatase fractions systematically: this decision has been made in agreement with Drs Dodgson and Spencer. The different effect of phosphate obtained in the two laboratories is therefore due to the use of different substrates and to the substrate specificity of the sulphatases (Dodgson *et al.* 1954*b*).

Sulphatase A. The effect of various electrolytes on the activity of sulphatase *A* is shown in Fig. 2. It should be noted that in these experiments a high concentration of enzyme was used so that the pH optimum was approximately pH 5·1 (Roy, 1953*b*). The most striking effect is obviously that of sulphate ions: in their presence the pH optimum is shifted from pH 5·1 to 6·2. Because of this shift in the position of the optimum there is an apparent activation of sulphatase *A* by sulphate ions on the alkaline side of the pH optimum of the untreated enzyme. It must be stressed that this phenomenon is not an activation in the true sense, as the activity of the sulphate-treated enzyme at the appropriate pH optimum is always less than that of the untreated enzyme under its optimal conditions (Fig. 2).

No comparable effect was noted with any of the other ions studied, suggesting that the mechanism of inhibition by sulphate is different from that of the other anions shown in Fig. 2. It is of interest that 0·08 M potassium chloride causes a slight (10 %) inhibition of sulphatase *A* at the pH optimum and on the alkaline side thereof: 0·005 M magnesium acetate caused a 10 % activation throughout the entire pH range studied. Previously reported experiments using high concentrations of magnesium chloride were presumably complicated by the simultaneous and opposing effects of magnesium and chloride ions.

Sulphatase B. In this case no comparable activation by sulphate ions was noted (Fig. 3), but chloride ions had a definite activating effect. It was reported previously (Roy, 1954*a*) that the pH optimum of sulphatase *B* was pH 5·7–5·8; these experiments were unfortunately carried out in the presence of variable amounts of chloride, and it has now been found that in the absence of chloride ions the pH optimum is in the region of pH 6·0 (Fig. 3). It can be shown by the use of chloride-free substrate solutions that chloride ions cause a pronounced activation on the acid side of the pH optimum with the net result of a broadening of the pH optimum into the acid region.

The influence of a number of other ions on sulphatase *B* activity is also indicated in Fig. 3. In contrast to sulphatase *A*, sulphatase *B* was uninfluenced by 0·005 M magnesium acetate.

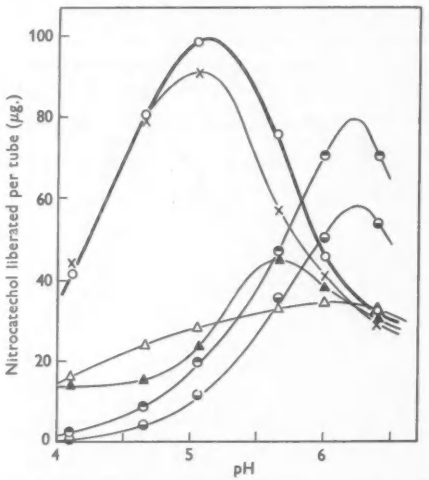

Fig. 2. Effect of electrolytes on the activity of purified sulphatase *A*. Conditions as in Fig. 1, except that the substrate concentration was 0·003 M nitrocatechol sulphate. ○—○, control; ×—×, 0·08 M-KCl; △—△, 0·0005 M-NaH_2PO_4; ▲—▲, 0·00001 M-Na_2SO_3; ◓—◓, 0·025 M-Na_2SO_4 or K_2SO_4; ◒—◒, 0·05 M-Na_2SO_4.

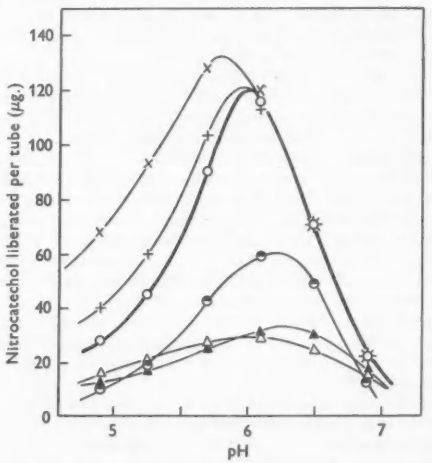

Fig. 3. Effect of electrolytes on the activity of purified sulphatase *B*. Conditions as in Fig. 1, except that the substrate concentration was 0·05 M nitrocatechol sulphate. ○—○, control; × — ×, 0·08 M-KCl; + — +, 0·01 M-KCl; △—△, 0·0025 M-NaH_2PO_4; ▲—▲, 0·0001 M-Na_2SO_3; ◓—◓, 0·1 M-Na_2SO_4.

Table 1. *The action of sulphydryl compounds and of* SH *reagents on sulphatases* A *and* B

Enzyme solutions treated for 1 hr. at room temp. with the inhibitors in 0·25 M acetate buffers, pH 5·0 and 5·9 for sulphatases *A* and *B* respectively. Reversal of inhibition by SH reagents attempted by addition of sulphydryl compound 10 min. before addition of the substrate.

Results of two experiments given, each being the mean of duplicate estimations, with an accuracy of ±2%.

Reagent	Concn. (M)	Activity (relative to control = 1)			
		Sulphatase *A*		Sulphatase *B*	
Iodoacetate	10^{-2}	0·27	0·30	0·05	—
p-Chloromercuribenzoate	10^{-4}	0·31	0·33	1·09	1·07
Cysteine	5×10^{-3}	1·01	1·08	1·25	1·19
Glutathione	5×10^{-3}	0·91	0·88	1·02	0·97
p-Chloromercuribenzoate + cysteine	10^{-4} + 5×10^{-3}	0·98	1·05	1·25	1·19
p-Chloromercuribenzoate + glutathione	10^{-4} + 5×10^{-3}	0·73	0·81	1·01	0·99

Miscellaneous inhibitors

There has been no report in the literature of any compound which might be regarded as a specific inhibitor of mammalian sulphatases, nor indeed of any sulphatase, except for the report by Robinson, Smith, Spencer & Williams (1952) that taka-sulphatase is inhibited by potassium 4-hydroxy-3-nitrophenyl sulphate. This ester is, however, hydrolysed by both sulphatases *A* and *B*. Torda (1943) claimed that the sulphatase of cat muscle was inhibited by 5% (w/v) cocaine and activated by 5% (w/v) yohimbine.

No result of any interest was obtained in the study of various alkaloids on sulphatases *A* and *B*, neither of which was affected by yohimbine (10^{-2} M), eserine (10^{-3} M), nor the methiodide of *m*-dimethylaminophenyl methyl carbamate (10^{-3} M). Cocaine (10^{-2} M) had likewise no effect on sulphatase *B*, but slightly inhibited sulphatase *A*; higher concentrations of cocaine strongly inhibited sulphatase *A*, but this effect would seem to be of no significance.

The effect of SH reagents is of some interest, and the results are shown in Table 1. Sulphatase *A* was strongly inhibited by iodoacetate and *p*-chloromercuribenzoate: the inhibition of the latter was reversed partially by glutathione and completely by cysteine. Cysteine alone had no effect on sulphatase *A*, but glutathione inhibited slightly. Some specimens of glutathione were very powerful inhibitors of sulphatase *A*: this effect varied from specimen to specimen, although all were more than 90% reduced, as judged by iodine titration, and were free from copper. It is likely that the inhibition was due to decomposition products, as the most inhibitory samples had a pronounced sulphurous smell. Sulphatase *B* was likewise inhibited by iodoacetate, but was slightly activated by *p*-chloromercuribenzoate; a sample of glutathione giving a 10% inhibition of sulphatase *A* was without action on sulphatase *B*, while cysteine activated strongly. The activation by *p*-chloromercuribenzoate was unexpected, as the other results in Table 1 would suggest that sulphatase *B*, like sulphatase *A*, was an SH enzyme. The explanation may be that the *p*-chloromercuribenzoate inhibited some agent in the enzyme preparation which tended to inactivate the sulphatase. In this connexion it should be noted that the available preparations of sulphatase *B* are probably much less pure than those of sulphatase *A*, the protein concentration in the reaction mixture being approximately 250 μg./ml. in the case of *B* and 20 μg./ml. in *A*.

The results, however, indicate that sulphatase *A* is an SH enzyme and are not incompatible with the view that sulphatase *B* is also an SH enzyme, although they do not prove the latter point.

DISCUSSION

The results resolve many of the conflicting reports in the literature and stress the need for using at least partly purified preparations in the study of enzyme inhibition. The activation of the sulphatase of a whole dispersion of ox liver by both sulphate and chloride ions under the appropriate conditions is further evidence for the view that such a preparation contains both sulphatases *A* and *B* which are therefore not artifacts.

In the case of the purified enzymes, one of the most interesting effects is the very powerful inhibition of sulphatases *A* and *B* by sulphite ions, the affinity of both enzymes for sulphite being approximately 100 times their affinity for sulphate and for nitrocatechol sulphate (Roy, 1954*a*). This perhaps reaches the extreme case in taka-sulphatase which, although strongly inhibited by sulphite (Robinson *et al.* 1952) is unaffected by sulphate. No explanation of this can be offered, but it is unlikely that the effect is due simply to the reducing power of sulphite ions, as cysteine is not an inhibitor. Neither does it seem probable, owing to the reversible nature of the inhibition by sulphite, that it is caused by the sulphite reacting with an aldehyde

grouping in the enzyme, as postulated by Rosenfeld & Ruchelman (1940) in the case of taka-sulphatase. The striking shift in the pH optimum of sulphatase *A* in the presence of sulphate is very interesting: it would seem that this must be due to combination of the sulphate with the enzyme, probably the active centre, as the inhibition of sulphatase *A* by sulphate is competitive (Roy, 1953*b*). This might be taken as further evidence that sulphatase *A* contains a positively charged grouping in its active centre (Roy, 1954*a*).

The general problem of the function of these enzymes remains obscure. The possibility that sulphatase might play a part in the *in vivo* synthesis of sulphuric acid esters by acting as a transferase with the 'activated sulphate' of Bernstein & McGilvery (1952) as substrate has been considered briefly by Dodgson *et al.* (1954*a*). The possibility of the occurrence of this reaction *in vivo* cannot be assessed, but it is clear that it could not be occurring in the *in vitro* experiments of Bernstein *et al.* (1952), as these workers used isotonic phosphate buffers, pH 6·5, under which conditions both sulphatases *A* and *B* are completely inactive, and sulphatase *C* only slightly active. It is likewise impossible to assess the possible hydrolytic activities of sulphatases *in vivo* as, according to Gamble (1947), the intracellular fluid contains 100 m-equiv./l. of phosphate, mostly organic. *In vitro* experiments indicate that sulphatases *A* and *B* would be strongly inhibited in such a medium, both enzymes being inhibited by phosphate esters. It is not possible to give quantitative figures owing to the difficulty of obtaining such esters completely free from inorganic phosphate, a very powerful inhibitor.

The multiplicity of sulphatases (Roy, 1953*a*; Dodgson *et al.* 1954*b*) and their widespread distribution throughout animal tissues (Rutenburg *et al.* 1952) might be taken to indicate that these enzymes are fulfilling some important metabolic role, Haldane (1954) even suggesting that such a multiplicity of enzymes might be an evolutionary adaptation of some advantage to the organism concerned. No such metabolic function is as yet obvious, the bulk of the evidence indicating a surprising inertness of sulphatases *in vivo* (Hawkins & Young, 1954). This situation invites comparison with that of β-glucuronidase which also exhibits this multiplicity (Mills, Paul & Smith, 1953) and apparent inertness *in vivo* (Garton & Williams, 1949). It may well be that both groups of enzymes play related roles in the metabolism of mucopolysaccharides, as suggested by the work of Meyer, Linker & Rapport (1951) for β-glucuronidase, and that their respective activities *in vivo* are only distantly related to the commonly used assay methods. In the case of sulphatases at least this would be borne out by the peculiar affinity of these enzymes for highly unphysiological substrates.

SUMMARY

1. Unfractionated preparations of ox-liver sulphatase are activated both by chloride and sulphate ions.

2. Sulphatase *A* is activated by sulphate ions on the alkaline side of the pH optimum owing to a shift of the position of the pH optimum in the presence of sulphate ions. A slight inhibition of sulphatase *A* is brought about by chloride ions at and above the pH optimum.

3. Sulphatase *B* is activated by chloride ions on the acid side of the pH optimum.

4. The effects of various other anions on sulphatases *A* and *B* are given.

5. Sulphatases *A* and *B* are uninfluenced by sodium and potassium ions; magnesium ions are without action on sulphatase *B*, but activate sulphatase *A* to a small extent.

6. Sulphatase *A* is shown to be an SH enzyme. In the case of sulphatase *B* the evidence is conflicting.

7. The significance of the influence of ions on sulphatases is discussed in relation to the possible activity of these enzymes *in vivo*.

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Mode of Insecticidal Action Studied with Labelled Systems

PHOSPHORYLATED COMPOUNDS IN THE MUSCLE OF THE ADULT HOUSEFLY, *MUSCA DOMESTICA*, L.

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Phosphorylated intermediates are involved in a ubiquitous and vital manner in nerve and muscle function. A disturbance in the normal distribution of these intermediates by an insecticide is likely, therefore, to be of significance in its mode of action. Disturbances in the distribution of phosphorylated intermediates in insect muscle have been studied by a combined radioactive tracer–paper chromatography technique. Insects were fed with radioactive phosphorus (^{32}P) so that the intermediates became labelled. The labelled compounds were extracted under conditions likely to preclude their decomposition and resolved by means of unidimensional paper chromatography. The paper chromatograms were then scanned radiometrically (Winteringham, Harrison & Bridges, 1952). In this way the ^{32}P-radioactivity (ordinate) was automatically plotted against distance along the paper chromatogram (abscissa) and the separated labelled fractions appeared as peaks of radioactivity. Provided the fractions so separated were isotopically labelled in a uniform manner the area enclosed by each peak of the radiochromatogram was proportional to the weight of labelled fraction present. By comparing radiochromatograms of normal and of poisoned tissues significant changes in the distribution of labelled metabolites can be determined. The absence of a change in distribution does not, of course, preclude any change in metabolic turnover rates. It seems likely, however, that any morbid changes in turnover rate would be reflected by a change in distribution. Some compounds, e.g. phosphoproteins, may not have become significantly labelled by the short-term feeding techniques used on account of relatively slow turnover rates. This paper is not concerned with these compounds. The techniques to be described are an attempt to study toxic action *in vivo* and to compare the results obtained with those which have been, or may be, predicted from experiments *in vitro*, e.g. with isolated enzyme systems. This paper describes the application of these techniques to the determination of the phosphorylated intermediates of the thoracic muscle of the adult housefly, *Musca domestica*, L. Later papers will describe the effects of insecticides on the distributions found. For a preliminary communication concerning this work see Winteringham, Loveday & Hellyer (1953).

EXPERIMENTAL

^{32}P-*labelled and unlabelled phosphorus compounds*

Adenosine triphosphate (*ATP*), *adenosine diphosphate* (*ADP*) *and muscle adenylic acid* (*AMP*). These were purchased as the sodium salt, the barium salt and as the free acid, respectively, from Light and Co.

Phosphoglyceric acid (*PGA*). This was purchased as the barium salt from Light and Co.

Ribose 5-phosphate (R 5-P) *and glucose 6-phosphate* (G 6-P). Samples of the barium salts supplied by the Schwarz Laboratories Inc., N.Y., were the gift of Mr C. J. Threlfall. Barium salts were converted into the sodium salts.

Glucose 1-phosphate (G 1-P). This was prepared as the potassium salt by Mr S. E. Lewis of this Laboratory, by the method of Sumner & Somers (1944).

^{32}P-*labelled glucose 6-phosphate.* Glucose was phosphorylated in the presence of inorganic phosphate and fresh baker's yeast (purchased locally from the Distillers Co. Ltd.) by the procedure of Neuberg & Lustig (1942), but the addition of fluoride was omitted and ^{32}P-labelled phosphate was substituted for ordinary phosphate. From the trichloroacetic acid (TCA) extract, free orthophosphate, PGA and hexose diphosphate (HDP) were separated as the magnesium and barium salts, respectively, by the method of Dubois & Potter (1943). The remaining solution which contained hexose monophosphates was freed of excess barium by sulphate precipitation and hydrolysed with N-HCl for 4 hr. at 100° to remove pyrophosphate and fructose 6-phosphate (F 6-P). Paper chromatographic analysis of the 'labelled' solution (see below) indicated the presence of two major fractions of R_F values respectively correct for G 6-P and PO_4^{3-}. The labelled G 6-P fraction was eluted from paper chromatograms and used as required.

^{32}P-*labelled phosphoglyceric acid.* Glucose was phosphorylated as before but fluoride was added to increase the yield of PGA. The labelled PGA was recovered as the barium salt (Dubois & Potter, 1943) and converted into the sodium salt.

^{32}P-*labelled hexose diphosphate.* This was isolated as the barium salt from the mixture used for preparing the PGA followed by conversion into the sodium salt. Paper chromatographic analysis indicated the presence of two compounds, the larger fraction behaving as HDP on the basis of

R_F value and rate of hydrolysis in N-HCl at 100°. It was eluted from paper chromatograms as required.

^{32}P-labelled pyro- and meta-phosphates. These were prepared by heating ^{32}P-labelled Na_2HPO_4 and NaH_2PO_4 at 250° and at red heat (Partington, 1933).

Incorporation of ^{32}P into the tissue phosphorus of adult houseflies in vivo

Housefly pupae were placed in breeding cages at 27° and provided with A.R. glucose and water *ad lib.* One- to five-day old adults which had been starved of food and drink for a few hr. were sexed while under mild *cyclo*propane anaesthesia and twenty-five females transferred to a 400 ml. Pyrex metabolism unit of the kind shown in Fig. 1. The B-50 cone (*C*) is closed at the top to form the floor of the unit. In the middle of this floor is a depression *B* or 'feeding-well' of about 1 ml. capacity. The well is charged with an absorbent wool plug moistened with 5% (w/v) A.R. glucose containing 1–1000 μc (according to the level of activity required) of carrier-free $^{32}PO_4^{3-}$ adjusted to pH 6 by the addition of $NaHCO_3$ to the sterilized solutions supplied by the Isotope Division of the Atomic Energy Research Establishment. Paper chromatographic analysis of the radioactive phosphate supplied showed that it consisted entirely of free orthophosphate. Occasionally a negligible trace of ^{32}P behaved as pyrophosphate. The metabolism unit was aerated (*D* and *E*) with dry CO_2-free air at 3 ml./sec. The flies were allowed to feed, walk or fly for a 'labelling' period of 24 hr. The dry atmosphere encouraged copious drinking during this period. After 24 hr. the flies were anaesthetized by substituting *cyclo*propane for air at *D* for 2 min. The feeding-well was then replaced by one containing no radioactive phosphorus and the flies kept for a further 'metabolism' period of 24 hr. During 'labelling' and 'metabolism', which were conducted at 30° in a lighted cabinet, the wells were kept visibly moist by the addition of 5% (w/v) A.R. glucose from the reservoir *A*. Dead insects were removed at the end of the 'labelling' or 'metabolism' period. Usually all the insects survived and behaved normally.

Dispersion and extraction of tissue

The thorax of the housefly bears the legs and wings and is very rich in muscle. Extracts of whole thoraces representing muscle extracts were prepared as follows. At the end of the 'metabolism' period the flies were anaesthetized in *cyclo*propane for 2 min. and placed inside a small stainless-steel wire cage which was plunged into liquid nitrogen. The cage and contents were then rinsed by brief immersion in 50% aqueous ethanol containing 5% (v/v) orthophosphoric acid at −10°. In this way external contamination by carrier-free ^{32}P (as a result of walking over the feeding-well, etc.) was effectively removed. Paper chromatographic analysis (see below) showed that free orthophosphate only was present in this external rinse. The removal of external phosphorus was of importance. It was unlikely to have undergone isotopic dilution by tissue phosphates so that its presence in the extract would appear as an artificially high proportion of labelled PO_4^{3-}. The cage and contents were then successively rinsed in 50% aqueous ethanol alone and in acetone both at −10°. The washed and chilled flies were then transferred to *iso*pentane at −70° to await dissection. Flies were transferred one at a time to a Petri dish chilled in solid CO_2. The heads, abdomens, and appendages were rapidly dissected off and the thoraces dropped into 1·8 ml. of N formic acid in 50% aqueous ethanol at −10° contained in a cooled all-glass 'homogenizer' of the type described by Potter (1949). Ten such thoraces were dispersed for 10 min. The dispersion was centrifuged at 5° and the supernatant stored at −15°. A further 1·8 ml. of ethanolic formic acid was added to the sediment and the dispersion repeated. This was repeated once more so that the pooled supernatants were almost exactly 5 ml. The total extract was concentrated to 1 ml. by subliming off the frozen solvent under high vacuum. The concentrate was finally centrifuged at room temperature and the slightly cloudy supernatant stored at −15°. The extracts contained acid-labile phosphorus compounds and for this reason the use of strong acids like TCA was avoided. The doubtless greater proportion of protein present as a result of this was without effect on the subsequent chromatographic separations. The presence of ethanol reduced the glycogen solubility and increased the solvent volatility for evaporation purposes.

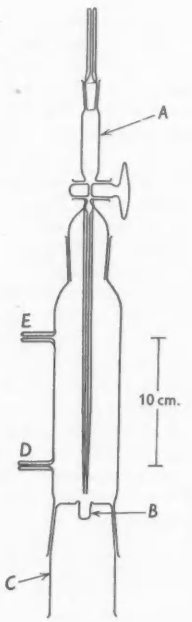

Fig. 1

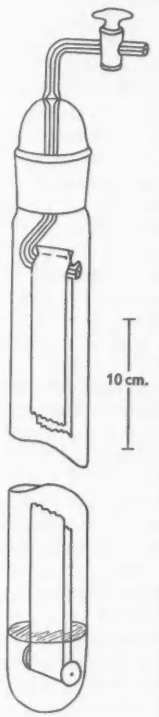

Fig. 2

Fig. 1. Metabolism chamber for feeding adult houseflies with ^{32}P-labelled phosphate solution. *A*, glucose reservoir; *B*, feeding-well depression in closed 'B-50' cone *C*; *D*, and *E*, inlet and outlet tubes for air circulation.

Fig. 2. Column for ascending paper chromatography under reduced pressure.

Finally formic acid was used in the principal solvent for chromatography so that its use for extraction avoided the presence of other acids which tend to interfere with the separations when present in the samples applied to paper chromatograms. Concentrates stored at −15° showed no changes in composition over several days. Assays of ^{32}P in the sediments (counted as suspensions) and in the extracts indicated that about 80% of the total tissue ^{32}P was recovered in the extracts. Most of this was recovered in the first 1·8 ml., which suggests that the residual ^{32}P was present as insoluble phosphoprotein, phospholipid, etc.

Paper-chromatographic techniques

Three of the solvents recommended by Burrows, Grylls & Harrison (1952) have been successfully adopted for separating the major phosphorylated intermediates of housefly muscle. The solvents used were made up by volume as follows: formic acid–acetone–water (14:60:26) (solvent no. 1); acetic acid–acetone–water (15:50:35) (solvent no. 2); 2N-NH_3–methanol (30:70) (solvent no. 3).

Whatman no. 1 paper, purchased in rolls of strip 1·125 in. wide, was used throughout. The paper was washed by soaking it overnight in 0·1N-HCl followed by successive rinsing in dilute ammonia and glass-distilled water. Washed 160 cm. long strips were folded double and pairs of samples (10–50 μl.) of the concentrate were applied at starting lines 7 cm. above the fold. The free ends of the paper were pinned together and a small glass weight was placed in the fold of the strip which was then hung in an all-glass column of the type shown in Fig. 2. Each column contained 100 ml. of the required solvent and the strip was immersed so that the starting lines were about 5 cm. above the solvent. The column was then exhausted at a water pump to ensure immediate saturation of the column with the solvent vapour. Ascending chromatography proceeded for 48 hr. at 5° for solvent 1 and at 25° for solvents 2 and 3. The solvent usually ascended 50 cm. or so above the starting lines. When strips were removed from the columns they were placed in a desiccator, dried *in vacuo* over soda-lime and charcoal, and stored at −15° until required for radiometric scanning (see below) or for chemical tests. After having located an incompletely resolved fraction by radiometric scanning, the paper containing it was cut out and attached to a new strip of paper. The fraction was then re-concentrated into a narrow band on the new strip by a simple elution technique (Winteringham, 1953). Except for the slowest running fractions complete re-concentration was achieved in 24 hr. at 5° in a mild draught. In the present work the eluting solvent consisted by volume of 0·1% redistilled formic acid in 50% aqueous acetone. The re-concentrated fraction was resolved in a second solvent by the procedures described.

The behaviour of a particular fraction under the conditions of acid hydrolysis was studied by suspending the paper section containing the fraction (marked off lightly in pencil during radiometric scanning) in acid at the required temperature. At intervals suitable samples were chromatographed in the usual way. In interpreting the subsequent radiochromatograms, account was taken of the acidity of the sample used. The presence of a strong acid invariably caused a fraction to run with a high R_F value.

Location of known compounds added to paper chromatograms. Unlabelled nucleotides were located and determined by neutron activation (see below) or by their inhibition of the fluorescence in ultraviolet light at 2540Å (Holiday & Johnson, 1949). Labelled compounds were located and determined by the radiometric scanning techniques described below. Unlabelled compounds were detected chemically on paper by the acetone-dip technique of Burrows *et al.* (1952), using a modification of the molybdate reagent of Hanes & Isherwood (1949).

Radiometric scanning of paper chromatograms. The ^{32}P-labelled fractions resolved by paper chromatography were located and assayed quantitatively by an automatic scanning technique which has been described in detail elsewhere (Winteringham *et al.* 1952). In the present work the fractions separated were isotopically labelled completely and apparently uniformly (see below) so that the relative areas enclosed by the peaks of the plotted radiochromatograms (measured by planimeter) represented the relative proportions in which the fractions were present.

The Geiger–Müller tubes used possessed 1 in. end-windows (2 mg./cm.^{2}). Tests with known amounts of ^{32}P-labelled PO_4^{3-} run on Whatman no. 1 paper indicated that about 20% of the ^{32}P-disintegrations were recorded. Geometrical factors were the principal cause of inefficiency, window absorption and self-absorption by the paper being negligible for ^{32}P.

Neutron activation of paper chromatograms. Neutron activation was employed (i) for estimating the relative $^{31}P/^{32}P$ ratios of the labelled fractions, (ii) for the detection of unlabelled phosphorus compounds, and (iii) for the estimation of the total phosphorus content of the extracts. Paper chromatograms of labelled or unlabelled extracts were irradiated in the Harwell pile (BEPO) at a neutron flux of 10^{11} neutrons/cm.^{2}/sec. for 120 hr. Great care was taken not to contaminate the strips (e.g. by fingering) before irradiation. In the present work the ^{32}P concentration varied from 10^{-2} to 10 μc/thorax (mean wt. of thorax was 7 mg.). This corresponded to $3{\cdot}4 \times 10^{-8}$ to $3{\cdot}4 \times 10^{-5}$ μg. of isotopic ^{32}P/thorax. The total weight of natural ^{31}P (natural isotopic abundance is 100%) was estimated by the activation of unlabelled extracts to be of the order of 10 μg./thorax. The labelling process therefore results in a negligible increase in tissue phosphorus. In practice 'carrier-free' ^{32}P may have undergone isotope dilution by a factor of 300 by phosphorus present in the irradiated target, reagents employed for extraction etc. (For this information the authors are indebted to Mr Brian Payne of the Isotope Divison, Harwell.) The increase in tissue phosphorus would still be less than 0·1%. It follows that the ratio of the net increase in ^{32}P activity on activation to the original ^{32}P activity due to labelling is a measure of the $^{31}P/^{32}P$ ratio in the separated fraction. It was calculated that 1 μg. of ^{31}P would give rise to about 6000 β-particles/sec. [by the ^{31}P (n, γ) ^{32}P-reaction] and that this could be assayed to within a few parts per cent by scanning a week or so after removal from the pile. This was confirmed experimentally by the irradiation of known weights of $Na_2HPO_4, 12H_2O$ run on paper chromatograms.

The irradiation of insect extracts resolved on paper chromatograms gave rise to radioactive isotopes other than ^{32}P. ^{24}Na and ^{42}K were identified by their energetic β- and γ-emission and by their short half-lives. It was interesting to note that these metals ran quite separately from the PO_4^{3-} as free cations in acid solvents when applied as the salts. Emitters of soft β-rays which had relatively long half-lives were also produced and probably consisted largely of ^{35}S arising by the ^{35}Cl (n, p) ^{35}S-reaction with chlorides doubtless

present in the extracts. Irradiated chromatograms were scanned 1 week after removing them from the pile by which time ^{24}Na and ^{42}K had decayed to insignificance. By scanning through a 28 mg./cm.^{2} aluminium screen the soft β-particles were effectively screened off. The remaining activity was due entirely to ^{32}P on the basis of half-life determinations over a period of 5 weeks.

Histological distribution of tissue ^{32}P

The autoradiographic technique described by Wineringham, Harrison & Hammond (1950) was used to study the histological distribution of ^{32}P in the fly thorax before extraction. In this way the danger of leaching by the autoradiographic technique itself was avoided.

Thoraces prepared for extraction were intercepted at the *iso*pentane stage and placed upon a degassed paraffin wax (m.p. 58°) platform contained in a short-path freeze-sublimation unit under high vacuum. The unit was submerged in a bath of ethyl oxalate slush at $-40°$ to $-50°$ (Mendelow & Hamilton, 1950). When dehydration was complete the tissue was embedded by melting the wax platform, blocked out, and sectioned at 10 μ. Consecutive sections were stained (Heidenhain's haematoxylin and eosin) and autoradiographed (Kodak fine grain stripping emulsion), respectively. A constant difficulty was that of cutting undamaged sections. The tissue was not fixed chemically and almost invariably the relatively brittle cuticle broke away. This has happened in the typical section shown in Fig. 4.

RESULTS

Qualitative and quantitative composition of the extract

The most useful initial chromatographic fractionation was obtained in solvent 1. A typical radiochromatogram obtained with this solvent is shown in Fig. 3. Five major fractions, I_1, II_1, III_1, IV_1 and V_1 (right subscript indicates the solvent number) were resolved. Fraction III_1 was sometimes partly resolved into two fractions but rarely sufficiently well for quantitative analysis (e.g. see Fig. 3). At least three distinct compounds were present in this fraction in any case. The major fractions ran with mean R_F values of 0, 0·15, 0·37, 0·57 and 0·70, respectively. Fraction II_1 could be resolved in solvent 2 into two fractions, I_2 and II_2 (the latter usually present in small proportion or absent) of mean R_F values 0·14 and 0·34, respectively. Fraction III_1 was further resolved by solvent 3 into three fractions, I_3, II_3 and III_3 of mean R_F values 0, 0·31 and 0·46, respectively.

These fractions have been tentatively identified by comparing their R_F values with those published for known compounds (Burrows *et al.* 1952), by co-chromatography with authentic compounds, and by their behaviour on acid hydrolysis. There is evidence that compounds other than those identified were present in small proportions but their identification was not considered practicable or necessary at the present stage of the work. When a fraction is described therefore as a particular compound it must be understood that this does not exclude the possible presence of other compounds in relatively small amounts.

Fraction I_1. This largely disappeared from extracts which were centrifuged at 6000 *g* for 60 min. before chromatography. It was insoluble in N-HCl at 100° when the paper containing the fraction was suspended in the acid for 7 min. It was concluded that I_1 was probably phosphoprotein in suspension and has not therefore been included in the total acid-soluble ^{32}P. Added ^{32}P-labelled metaphosphate remained at R_F 0 in solvent 1 but clearly was absent from fraction I_1 because of the relative ease with which metaphosphate undergoes hydrolysis.

Fraction II_1. Added ATP or ADP ran in this fraction, the latter being the faster when they were added to the sample prior to chromatography in solvent 1. Hydrolysis of fraction I_2 (prepared by resolving II_1 in solvent 2) for 10 min. in N-HCl at 100° liberated two-thirds of the ^{32}P which ran as PO_4^{3-} in solvent 1. Added ATP co-chromatographed exactly with I_2. Hydrolysis of II_2 for 10 min. in N-HCl at 100° liberated one-half of the ^{32}P as PO_4^{3-}. Added ADP ran with II_2. The hydrolysis of the combined fraction II_1 (I_2+II_2) for 10 min. in N-HCl at 100° gave rise to PO_4^{3-} and a major derivative which co-chromatographed exactly with added R 5-P in solvent 1, the combined fraction running with an R_F value of 0·50. A minor derivative co-chromatographed exactly with AMP under the same conditions. II_1 was selectively hydrolysed in the total extract in the presence of a suitably buffered potato apyrase prepared according to Lee & Eiler (1951). It was concluded that I_2 and II_2 were respectively ATP and ADP and that the α-, β-, and γ-P atoms were uniformly labelled. On a routine basis II_1 was used as an estimate of the total ATP + ADP. Resolution of II_1 in solvent 2 was used for estimating the ATP/ADP ratio (usually very high). A check on this ratio and on the uniformity of labelling was made by the hydrolysis of II_1 (after reconcentrating) for 10 min. at 100° in N-HCl followed by a determination of the ratio

$$\frac{^{32}P \text{ as } PO_4^{3-}}{^{32}P \text{ as AMP} + \text{R 5-P}}.$$

Fraction III_1. Labelled or unlabelled G 6-P co-chromatographed exactly with the faster running peak of III_1 (see Fig. 3). The added G 6-P ran exactly with III_3 (prepared by resolving III_1 in solvent 3). Labelled HDP and G 1-P ran either just too fast or too slowly in solvent 1 to suggest their normal presence in III_1. Labelled pyrophosphate ran with III_1 and remained at R_F 0 with I_3 in solvent 3. However, the rate of hydrolysis of none

of the separated components of III_1 corresponded to that of pyrophosphate, and its presence in III_1 in significant amount was ruled out.

Fraction I_3 (separated from III_1 in solvent 3) was slowly hydrolysed in 0·03 N-HCl at 28° but not in the presence of 0·30% ammonium molybdate. This suggested the presence of the invertebrate phosphagen, arginine phosphoric acid (Arg-P), in I_3 (and therefore in III_1) and the absence of creatine phosphoric acid (Meyerhof, 1928). I_3 was completely hydrolysed to PO_4^{3-} in 0·01 N-HCl at 37° in 4 hr. This also suggested that I_3 was Arg-P and not G 1-P (Meyerhof & Lohmann, 1928; Mende & Chambers, 1953). Free arginine was apparently present in the extract but ran faster than III_1 under the same conditions. Hydrolysis of III_1 invariably gave rise to a positive ninhydrin-reacting substance which, under the paper chromatographic conditions for separating arginine from the majority of amino acids (aqueous acetone solvent of Bentley & Whitehead, 1950) behaved as arginine. The formation of free arginine on hydrolysing III_1 was confirmed by Weber's (1930) modification of the Sakaguchi reaction for guanidine derivatives. It was concluded that I_3 was Arg-P. Mild hydrolysis of III_1 indicated that Arg-P ran with the slower peak of III_1.

AMP ran with the slowest-running part of III_1 in solvent 1. It appeared to co-chromatograph exactly with II_3 when the combined fraction (III_1 + AMP) was run in solvent 3. When this combined fraction was re-run in solvent 1 it was clear however that part of II_3 was not AMP. This was apparently due to the presence of an unidentified compound in II_3 (provisionally designated U.C. 1). In some experiments in which the ATP was apparently decomposed *in vivo* (e.g. as a result of methyl bromide poisoning), the slower running part of III_1 and II_3 (separated from III_1) was largely increased at the expense of II_1. II_3 then co-chromatographed exactly with AMP in solvent 3 and on re-running the combined fraction in solvent 1. U.C. 1 was now apparently completely masked by the AMP which clearly constituted most of II_3 in these circumstances. Hydrolysis of II_3 for 3 hr. in N-HCl at 100° liberated 30–60% of the ^{32}P-activity as PO_4^{3-}. The presence of two unidentified derivatives (R 5-P?) was indicated. Similar patterns of hydrolysis products were obtained whether II_3 was separated from normal extracts or from extracts in which the ATP had largely decomposed *in vivo* as above. This suggested that U.C. 1 might be related to AMP. Hydrolysis of III_3 for 3 hr. in N-HCl at 100° liberated 4–16% of the ^{32}P as PO_4^{3-}. This was consistent with the co-chromatographic experiments which strongly indicated that III_3 and G 6-P were identical.

It was concluded that III_1 consisted of Arg-P, AMP, G 6-P and an unidentified compound U.C. 1. On a routine basis III_1 has been used for estimating the combined fraction and resolution in solvent 3 for estimating the separated fractions Arg-P (I_3), AMP + U.C. 1 (II_3) and G 6-P (III_3).

Fraction IV_1. This fraction co-chromatographed exactly with unlabelled or ^{32}P-labelled PGA in solvent 1 and the combined fraction was not resolved on re-running in solvent 3. The chromatographic tests in solvent 3 were not entirely satisfactory. The added 'PGA', though unresolved in solvent 1 and co-chromatographing exactly with IV_1, gave rise to two or more fractions (located chemically) when re-run in solvent 3. However, one of these (R_F 0·62) co-chromatographed exactly with the eluted IV_1. Fraction IV_1 was completely stable to 3 hr. hydrolysis in N-HCl at 100°. Bandurski & Axelrod (1951), using an acid solvent similar to solvent 1, found that PGA ran 0·79 times as fast as PO_4^{3-}, which compares well with the value 0·81 found for fraction IV_1 in the present work. It was concluded that fraction IV_1 was PGA.

Fraction V_1. This fraction co-chromatographed exactly with ^{32}P-labelled orthophosphate or inactive phosphate. On re-running in solvent 3, fraction V_1 remained at R_F 0, but Burrows *et al.* (1952) reported an R_F value of 0·31 for sodium orthophosphate. This anomaly was apparently due to the fact that in the present work the phosphate

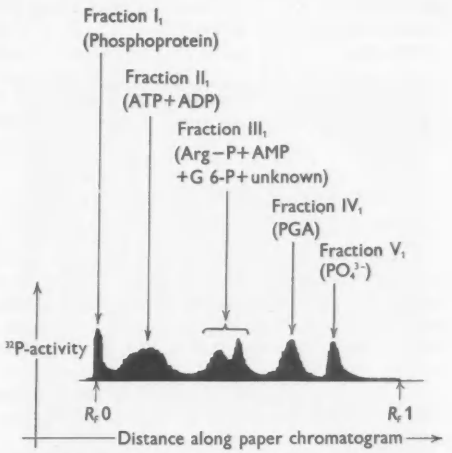

Fig. 3. Tracing of an original radiochromatogram illustrating the primary paper-chromatographic fractionation in formic acid–acetone solvent (solvent 1) of ^{32}P-labelled compounds extracted from housefly thorax. Peaks of radioactivity have been inked in for clarity. Fraction II_1 may be resolved in acetic acid–acetone solvent (solvent 2). Fraction III_1 may be resolved in ammonia–methanol solvent (solvent 3). The tentative identification of the fractions is indicated in the figure.

Table 1. *Distribution of* ^{32}P *in ethanolic–formic acid extracts of thoracic tissues of* Musca domestica *adults*

Fraction	^{32}P of fraction as % of the total soluble ^{32}P	Range	Remarks
ATP	37·2	31·7–46·8	ADP; 0–10%. By solvent-2 analysis
ADP	1·2		
Arg-P	10·9	18·6–30·8	Arg-P by solvent-3 analysis (13·4% by hydrolysis)
AMP + U.C. 1	6·9		
G 6-P	7·8		
PGA	20·8	18·5–24·0	—
PO_4^{3-}	15·2	10·6–21·1	—
	100·0		

was applied by elution of the free PO_4^{3-} ion separated first in an acid solvent, the eluted PO_4^{3-} then being held back in solvent 3 presumably by trace metals of the paper or extract (magnesium, calcium, etc.). Fraction V_1 was selectively precipitated from the extract by magnesium acetate at pH 8·2 although variable proportions of the ATP fraction were co-precipitated (cf. Lee & Eiler, 1953). Fraction V_1 was unchanged by hydrolysis and was concluded to be free PO_4^{3-}.

The tentative identification of the principal fractions is indicated in Fig. 3.

Mean composition of muscle extract. On the basis of this tentative identification the mean composition of several extracts made over a period of 3 months from groups of adult flies of different generations together with the ranges of variation found are summarized in Table 1.

Replicate analyses made on the same extract usually agreed to within 2% (total ^{32}P) of the mean value for each major fraction separated in solvent 1 provided the fractions were reasonably well resolved.

Uniformity of labelling; total acid-soluble phosphorus

It was possible that the phosphorylated intermediates extracted were not uniformly labelled isotopically. For example, Flock & Bollman (1944) found a much more rapid incorporation of ^{32}P into the terminal phosphorus atom (P^{γ}) of ATP than into the P^{α} and P^{β} atoms. In the present work the nucleotide was apparently uniformly labelled. An estimate of the relative $^{31}P/^{32}P$ ratios of the separated fractions was made by neutron activation of paper chromatograms of labelled extracts. This ratio for the PO_4^{3-} fraction could not be used for comparison because the unavoidable contamination of the labelled extract by inactive phosphate during the external rinse of the flies in dilute phosphoric acid gave an artificially high $^{31}P/^{32}P$ ratio in this fraction. The average results of two experiments in which labelled strips were allowed to decay and then activated are summarized below.

The results indicate the presence of a higher proportion of ^{31}P in the original nucleotide fraction than in the others. It is significant, however, that in the ADP and other fractions the $^{31}P/^{32}P$ ratios are in reasonable agreement. If in fact the high $^{31}P/^{32}P$ ratio of II_1 were due to incomplete labelling of the nucleotide fraction, the highest ratio would have been expected in the ADP fraction, because of its higher P^{α} content. It was concluded that while there was no evidence of incomplete labelling of the nucleotide in these experiments there was evidence for the presence of an unlabelled phosphorus compound in the nucleotide fraction behaving as ATP in solvents 1 and 2.

Limited acid hydrolysis of the resolved ATP and ADP fractions, respectively, liberated two-thirds and one-half the total ^{32}P-activity as free PO_4^{3-}. This would only be expected for uniformly labelled nucleotides. There were no differences in the proportions of the total ^{32}P-activity running as ATP in groups of flies which had been allowed to metabolize 24, 48, 72 and 120 hr., respectively. This also suggests that isotopic equilibrium among the labelled intermediates was achieved 24 hr. after labelling.

As a precautionary measure the nucleotide fractions have been hydrolysed on a routine basis and the fraction of total ^{32}P-activity liberated as PO_4^{3-} used as a check on the uniformity of labelling. Evidence of incomplete labelling would be taken into account in the final analysis and reported.

Fraction	II_1	I_2	II_2	III_1	IV_1
	(ATP + ADP)	(ATP)	(ADP)	(Arg-P + AMP + G 6-P + U.C. 1)	(PGA)
$^{1}P/^{32}P$ ratio: (that of 'PGA' taken as unity)	1·2	1·3	0·9	0·9	1·0

Histological distribution of ^{32}P

Since an extract of whole thorax has been used as an indicator of the distribution of phosphorylated intermediates of muscle it was of interest to determine the histological ^{32}P-distribution in transverse sections of the thorax. A typical stained transverse section and the corresponding autoradiograph made from the neighbouring section are shown in Figs. 4 and 5. ^{32}P-Concentrations appear as dark zones in the autoradiograph. A fairly uniform distribution of ^{32}P can be seen in the indirect flight muscle blocks in the upper half of the autoradiograph (*A*) while a relatively higher but localized concentration occurs in the walls of the stomach and proventriculus seen as elliptically shaped organs in the lower half of the autoradiograph (*B*). The higher concentration in the gut was confirmed by dissecting out gut and muscle separately from a labelled insect under cooled light petroleum. The tissue was dried and weighed on tared cover slips and the ^{32}P-activity assayed by direct counting under a thin end-window Geiger–Müller tube. The ^{32}P-activities of gut and muscle tissue gave respectively 75 and 30 net counts/min./μg. dry tissue. A pair of extracts was made of a horizontally bisected thorax so that one extract was of dorsal tissue rich in muscle and the other of ventral tissue containing the gut, ventral nerve ganglia, etc. Chromatographic analyses showed that these extracts were of very similar composition with some evidence of a higher ATP content of the gut-containing tissue. It was concluded that the use of extracts of entire thorax fairly represented the distribution of phosphorylated intermediates in the thoracic muscle.

Radiation and age effects

No attempt has been made to calculate the radiation dose in röntgen units received by the insects because of the difficulty of defining the geometrical conditions. A fly was not only exposed to radiation by self-absorption of the beta particles of its own tissue but to radiation from neighbouring flies, from the feeding-wells, and to that back-scattered by the walls of the metabolism unit. Tissue concentrations of ^{32}P were calculated, however, from the results of calibrating the scanning unit with known amounts of ^{32}P separated on paper chromatograms. These concentrations were compared with the distribution of labelled intermediates determined in seventeen different groups of ten flies in which the calculated ^{32}P concentrations varied from 0·002 μc to 1·01 μc/mg. wet tissue at the time of 'labelling'. There was apparently no relation between the small variations in distribution found and the radiation levels as indicated by the concentration of ^{32}P in the tissue. The ages of the same fly groups was either 3 days or 6–7 days at the time of extraction. The mean (ATP + ADP)-value (% total ^{32}P) was higher (42·2 %) for the older flies than for the 3-day flies (36·9 %) but the difference was not significant ($P > 0{\cdot}05$). It was concluded that any radiation or

Fig. 4

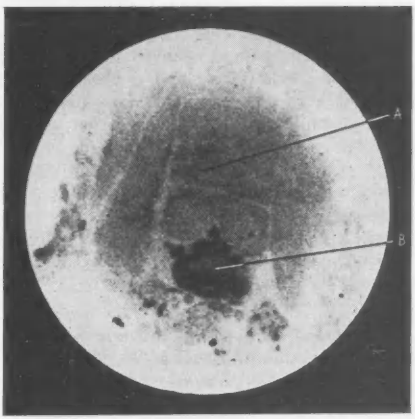

Fig. 5

Fig. 4. Photomicrograph of transverse section through thorax of adult housefly stained in Heidenhain's haematoxylin. *A*, indirect flight muscle; *B*, gut. (×25).

Fig. 5. Autoradiograph showing histological distribution of ^{32}P in transverse section through thorax of adult housefly (cf. Fig. 4). *A* corresponds to indirect flight muscle; *B* corresponds to gut. Darker zones indicate higher ^{32}P concentrations. (×25).

Table 2. *Distribution of labelled intermediates in thoracic muscle of flies at different levels of motor activity and* cyclo*propane anaesthesia*

All figures are % of total soluble ^{32}P

State of test flies at instant of death	Full flight		Crawling only		1 hr. continuous anaesthesia		5 hr. continuous anaesthesia	
Fraction	Control	Test	Control	Test	Control	Test	Control	Test
ATP + ADP	38·0	51·6	46·4	45·5	29·2	27·0	36·0	29·4
Arg-P + G 6-P + AMP + U.C. 1	19·4	8·6	20·4	15·6	27·4	30·6	18·0	30·7
PGA	31·4	10·2	18·0	11·4	32·7	22·7	34·2	14·7
PO_4^{3-}	11·2	29·6	15·2	27·5	10·7	19·7	11·8	25·2
	100·0	100·0	100·0	100·0	100·0	100·0	100·0	100·0

age effects were small compared to the natural variation in phosphorus distribution from one fly group to another.

Effects of cyclo*propane anaesthesia*

To bring all the insects used in these studies to a common state of rest they were anaesthetized in *cyclo*propane for 2 min. immediately before chilling, extracting, etc. Possible effects of *cyclo*propane were investigated by determining the distribution of labelled intermediates of 'test' groups of flies which were at different levels of motor activity or anaesthesia at the instant of death (plunging into liquid nitrogen). The results are summarized in Table 2. 'Controls' refer to groups of flies from the same generation, 'labelled' and 'metabolized' simultaneously with the test flies and subjected to the standardized 2 min. anaesthesia.

It will be seen that motor activity is associated with a reduction in the Arg-P fraction and increase in PO_4^{3-}. The fraction (ATP + ADP) during full flight was the highest ever recorded and therefore significant. Other effects believed to be significant are the increase in PO_4^{3-} and depletion in PGA during activity or extended anaesthesia. The evident accumulation of and depletion of Arg-P with relatively constant ATP during rest and activity respectively are consistent with current views of muscle biochemistry (Mommaerts, 1951; Greenberg, 1952). Finally the results suggest that the brief *cyclo*propane anaesthesia normally used causes no abnormal distribution of labelled intermediates.

DISCUSSION

Meyerhof & Lohmann (1928), Meyerhof (1928), and Needham, Needham, Baldwin & Yudkin (1932) have shown that the phosphagen of many invertebrate species is arginine phosphoric acid (Arg-P). Baldwin & Needham (1934) found Arg-P in the thoracic tissue of *Calliphora* and *Lucilia* species of blowfly. The presence of Arg-P as well as free arginine in thoracic tissue extracts of the adult housefly *Musca domestica* has been demonstrated in the present work. It is therefore surprising that Pratt (1950) was unable to detect free arginine in the haemolymph of *Musca domestica* adults. Insect and mammalian ATP appear to be identical (Albaum & Kletzkin, 1948; Calaby, 1951). The other phosphorylated intermediates of housefly muscle suggested by the present work are well-known extractives of mammalian muscle, and it is interesting to compare the overall distribution found with that reported by LePage (1948) for resting rat muscle. This has been done in Table 3. The original figures of LePage, which were in μmoles of intermediates per 100 g. of wet tissue, have been converted into g. of equivalent phosphorus per 100 g. of total acid-soluble phosphorus accounted for.

Table 3. *Comparison of phosphorylated intermediates found in the resting muscle of the adult housefly (present work) and in the rat (LePage,* 1948)

Compound	Phosphorus of compound expressed as % of total acid-soluble phosphorus: Housefly	Rat
ATP	37·2	33·6
ADP	1·2	2·4
Phosphagen	10·9	33·7
AMP	<6·9	3·2
G 6-P	7·8	5·2
PGA	20·8	2·9
PO_4^{3-}	15·2	15·3
Other compounds	>0·0	3·7
	100·0	100·0

Notable are the relatively low phosphagen and high PGA of the insect. Meyerhof (1928) found as much as 75% of the total acid-soluble phosphorus present as Arg-P in resting Crustacean muscle.

SUMMARY

1. The adult housefly, *Musca domestica* L., has been fed with carrier-free $^{32}PO_4$. The radioisotope was incorporated into the acid-soluble phosphorylated compounds of thoracic tissue. These compounds were extracted and separated on paper chromatograms and assayed by an automatic radiometric scanning technique.

2. The major fractions separated have been tentatively identified as ATP, ADP, AMP, glucose

6-phosphate, arginine phosphoric acid, phosphoglycerate and free orthophosphate.

3. Evidence was obtained that all the P atoms of the ATP and ADP fractions were equally labelled.

4. The histological distribution of the ^{32}P was studied by an autoradiographic technique. A relatively high concentration of ^{32}P in the gut of the thorax was found but appeared to be of the same chemical composition as that of the muscle.

5. The effects of motor activity and *cyclo*propane anaesthesia upon the distribution of labelled intermediates appeared to be consistent with current views in muscle biochemistry.

6. The phosphorylated compounds of resting housefly and mammalian muscle have been compared. Relatively low phosphagen and high phosphoglycerate levels in the insect were noted.

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The Biological Action of Substances Related to Thyroxine

10. THE DE-IODINATION OF THYROXINE IN THYROIDECTOMIZED RATS*

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In previous communications (Wilkinson & Maclagan, 1953, 1954; Wilkinson, Sprott, Bowden & Maclagan, 1954) it has been shown that, in intact rats, the output of urinary iodide after a single injection of ^{131}I-labelled thyroxine or triiodothyronine is considerably reduced by the simultaneous administration of butyl 4-hydroxy-3:5-diiodobenzoate (BHDB). In contrast with these results, Roche, Deltour & Michel (1953) reported that, in thyroidectomized rats, BHDB brought about an increase in the urinary iodide excretion after a single dose of radioactive thyroxine. Our work has therefore been extended to thyroidectomized animals in an endeavour to explain these conflicting results.

A brief report of some of the results has been published elsewhere (Wilkinson & Feetham, 1954).

* Part 9, Wilkinson & Maclagan (1954).

MATERIALS AND METHODS

Materials. Thyroxine labelled in the 3′:5′ positions with ^{131}I, purchased from the Radiochemical Centre, Amersham, was prepared for injection as described by Maclagan & Wilkinson (1954*a*). Immediately before use, the purity of the solutions was checked by paper chromatography, the thyroxine being located on the chromatograms by the method of Bowden & Maclagan (1954) or by autoradiography.

BHDB was administered in suspension (50 mg./ml.) in 1% (w/v) aqueous carboxymethylcellulose.

Animals. Male rats, weighing 160–180 g., were thyroidectomized as described by Gross & Pitt-Rivers (1953). After recovery from the operation the rats were checked for completeness of removal of the gland by comparing their weights with those of control animals and also by measuring individually their rates of O_2 consumption by the method of Maclagan & Sheahan (1950). Eleven days after operation each rat consumed 0·81–1·13 ml. O_2/g./hr., which compares with control values in the range 1·4–1·6 ml. O_2/g./hr. All rats which gained more than 10 g. in weight during the 15 days before the beginning of the excretion experiments were rejected.

Some confirmation of the completeness of the thyroidectomies was obtained from the fact that, of the first batch of twenty-four animals used in a preliminary experiment, all but seven died during the 4th week after operation. The deaths occurred during a spell of wintry weather when the temperature of the animal room fell by about 5°. The survivors, obviously affected by the cold, were maintained on thyroxine (approx. 2 μg./rat/day, as recommended by Barker *et al.* 1950), after which they recovered. Subsequently, the excretion experiments were carried out during the 3rd week after operation.

When the excretion measurements had been completed, twelve of the animals were maintained on thyroxine for 3 weeks, after which no radioactivity could be detected in the excreta. They were then placed on a low-iodine diet (oatmeal and tap water) for 4 days. Each rat was then given a single subcutaneous injection of carrier-free $Na^{131}I$ (5 μc.) and, 4 hr. later, was killed with chloroform. The trachea in the thyroid region was dissected, weighed, and dissolved in 0·4 N-NaOH (10 ml.). The radioactivity of the resulting solutions was measured in a liquid counter. The tracheal uptake was less than 0·4% of the injected dose except in three cases when it was about 1%, whilst similar measurements with control animals gave thyroid uptakes of over 10%. In view of the fact that these measurements were made 6 weeks after thyroidectomy it is reasonable to assume that some measure of thyroid regeneration had occurred in the rats showing 1% iodine uptake.

Excretion experiments. Groups of three thyroidectomized rats were given BHDB (50 mg./rat) orally on the first day of the experiment and 25 mg./rat on each succeeding day. Labelled thyroxine (2·5–20 μg./rat) was injected subcutaneously on the second day. Further groups received thyroxine only.

Each group was kept in a metabolism cage from which the urine and faeces were collected daily. The excreta were treated as already described (Wilkinson *et al.* 1954) and the radioactivity was measured.

RESULTS

The effect of BHDB on the output of urinary radioactivity after the injection of a single 5 μg./rat dose of ^{131}I-labelled thyroxine in thyroidectomized rats is shown in Fig. 1. It will be observed that it has produced a substantial reduction similar to that found in intact rats (Wilkinson & Maclagan, 1953; Wilkinson *et al.* 1954). Comparable results were obtained when 20 μg./rat doses of thyroxine were given.

Since the timing of the administration in these and the experiments with intact rats differed from that employed by Roche *et al.* (1953), an experiment was performed using their conditions. BHDB (25 mg./rat) was given orally on each of the 3 days immediately preceding the thyroxine injection. Of the two groups of three rats treated with BHDB, one showed an appreciable reduction in the excretion of urinary radioactivity, whilst the output of the other was indistinguishable from that of the control groups. The rats in this group, however, were constipated during the first day of the collection.

Further experiments were therefore performed in which BHDB was given on the day preceding the thyroxine injection and on the next 2 days only. The results obtained appear in Fig. 2. During the first 2 days after the administration of thyroxine the urinary radioactivity of the treated groups was much reduced as compared with that of the controls. Subsequently, however, the output increased until after 5 days it exceeded that of the controls. The change in the rate of excretion coincided with the onset of severe constipation in both BHDB-treated

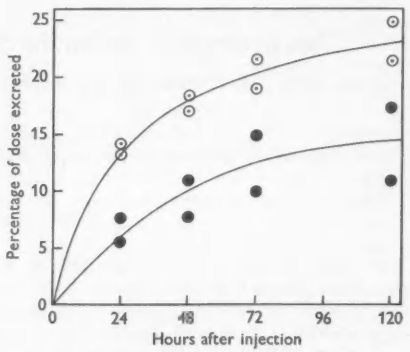

Fig. 1. The effect of BHDB on the urinary radioactivity of thyroidectomized rats treated with a single subcutaneous injection of ^{131}I-labelled thyroxine (5 μg./rat). ⊙, the combined output of a group of three rats treated with thyroxine alone; ●, the combined output of three rats treated with thyroxine + BHDB (see text for dosage).

groups. During the 48 hr. (3–5 days) each group of three rats produced only about 0·5 g. faecal pellets, instead of the normal 10–20 g./day.

The results of the faecal measurements in this and earlier experiments are summarized in Table 1. It will be observed that in each case where a low faecal output is recorded, severe constipation appears to be the immediate cause. This seems to be more liable to occur when BHDB is administered together with minimal thyroxine dosage.

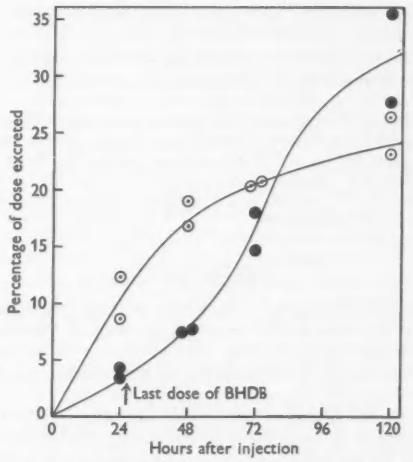

Fig. 2. The effect of stopping treatment with BHDB on the urinary radioactivity of thyroidectomized rats given a single subcutaneous injection of ^{131}I-labelled thyroxine (2·5 μg./rat). ⊙, the combined output of a group of three rats treated with thyroxine alone; ●, the combined output of a group of three rats treated with thyroxine + BHDB (see text for dosage).

DISCUSSION

Since it has been established that the urinary radioactivity of rats treated with thyroxine labelled in the 3′:5′ positions with ^{131}I is almost entirely due to iodide (Wilkinson *et al.* 1954), it follows from the results shown in Fig. 1 that BHDB reduces the output of urinary iodide. Consequently, it may be concluded that it interferes with the de-iodination of thyroxine in thyroidectomized animals in much the same way as in intact rats.

A possible explanation may be suggested to account for the surprising results of Roche *et al.* (1953) who, using one group of three rats, found exactly the opposite effect. Their rats were treated with BHDB on each of the 3 days preceding the thyroxine injection only. It is therefore probable that the concentration of the drug in the tissues after the thyroxine was given would be too low to exert any inhibitory effect, for it has been shown that, in the rabbit, BHDB is very rapidly excreted (Maclagan & Wilkinson, 1954*b*). In our experiments however, BHDB was given daily before and after the administration of thyroxine so as to ensure an adequate concentration in the tissues throughout the experiment.

This difference in timing of the BHDB treatment might well account for the absence of a reduction in the urinary iodide output in the experiments of Roche *et al.*, but it could hardly explain the increase they reported. We therefore decided to vary the

Table 1. *The effect of changes in the timing of the oral administration of BHDB on the faecal radioactivity of thyroidectomized rats treated with* ^{131}I*-labelled thyroxine*

Each row of figures refers to the combined excretion of a group of three rats. Dosage of BHDB: *A*, 25 mg./rat on each of 3 days preceding that on which the thyroxine was injected; *B*, 50 mg./rat on day before thyroxine injection, and 25 mg./rat on each of next 2 days; *C*, 50 mg./rat on day before thyroxine injection and 25 mg./rat daily throughout the experiment. '+' indicates that rats were severely constipated.

Dosage of		Percentage of dose excreted					
		In faeces after (hr.)					In faeces and urine after 120 hr.
Thyroxine (μg./rat)	BHDB	24	48	72	96	120	
2·5	None	27·4	43·8	51·9	—	57·9	80·9
		17·2	30·3	40·2	—	54·9	81·3
2·5	*A*	1·4+	20·2+	—	27·3+	28·8+	50·9
		18·7	28·1	—	36·8+	38·3+	48·3
2·5	*B*	19·5	26·7	32·8+	—	33·4+	69·2
		22·4	29·7	32·9+	—	39·8+	67·5
5	None	18·0	39·8	50·7	—	55·3	76·7
		24·1	39·2	50·2	—	56·1	80·5
5	*C*	31·0	51·4	57·6	—	61·5	77·8
		26·5	47·4	55·7	—	59·0	69·9
20	None	29·2	46·1	—	52·2	52·7	78·6
20	*C*	36·4	48·2	—	54·7	55·7	71·0

timing of the BHDB treatment and first repeated their experiment in duplicate. One group showed no decrease of urinary iodide output, whilst the other showed appreciable diminution. The effect of conditions intermediate between those used in our experiments and those of the French workers was investigated in another experiment in which the BHDB treatment was stopped 24 hr. after the thyroxine injection. During the first 2 days the inhibitory effect of BHDB was evident (Fig. 2), but later the iodide output increased in a manner similar to that reported by Roche *et al.*

The change in the rate of iodide excretion in this experiment coincided with the onset of severe constipation in both groups of BHDB-treated rats. Instead of the normal 10–20 g./day of faeces these rats excreted less than 0·5 g./day. The constipation appeared to be due to loss of appetite rather than to a direct effect of the withdrawal of BHDB. A study of the faecal radioactivities (Table 1) suggests that small doses of thyroxine (2·5 μg./rat) were insufficient to overcome the systemic effects of 3 days' treatment with BHDB, whilst larger doses of thyroxine were capable of preventing loss of appetite and the resulting constipation.

In a relative degree of intestinal stasis, thyroxine and its metabolic products excreted in the bile would undergo a much greater degree of re-absorption than in a normal gut. These re-absorbed substances would then be subjected to further de-iodination and more iodide would be available for excretion by the kidney. Thus the net result of severe constipation would be the diversion of radio-activity to the urine. Such an effect might account for the results of Roche *et al.* (1953) for in a footnote they report that in their experiments the faecal radioactivity was minimal.

We therefore suggest that BHDB reduces the rate of de-iodination of thyroxine in thyroidectomized as well as in intact rats, but the use of urinary iodide output as a measure of this process is only possible when the administration of the BHDB is correctly timed and when complications due to constipation do not occur.

SUMMARY

1. The output of urinary iodide by thyroidectomized rats after the injection of [3′:5′-^{131}I]thyroxine was substantially reduced by the simultaneous administration of butyl 4-hydroxy-3:5-diiodobenzoate.

2. A probable explanation of some conflicting results reported in the literature is given.

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The Hydration of the Cornea

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In the dead eye the cornea swells, at the same time losing its transparency; similar changes are observed when the isolated cornea is kept in physiological saline. These, and other, observations suggest that the cornea, *in vivo*, has a constant tendency to absorb water from its surrounding fluids, a tendency that is opposed by dehydrating factors which cease to operate on the death of the animal. Essentially, the cornea consists of a number of collagenous laminae—the stroma—separated from the tears by the epithelium, and from the aqueous humour by the endothelium (Fig. 1); it is only at the limbus, through the conjunctival capillaries, that the cornea comes into direct relationship with the blood. Under the electron microscope the structural units of the laminae are

visible as banded collagen fibrils; these are finer than those in tendon and sclera, and are heavily embedded in a ground substance that is probably mucoid (Schwarz, 1953). It is probably the high concentration of mucoid in the cornea that determines its transparency, and also its remarkable tendency to post-mortem swelling, both these characteristics being absent from the sclera. In the living eye, fluid and salt exchanges are theoretically possible across the epithelium and endothelium, and also, in the limbal region, across the limbal capillaries, so that turgescence of the cornea may conceivably result from the absorption of tears, aqueous humour, and limbal interstitial fluid. The cause of the turgescence would be the colloid-osmotic pressure exerted by the colloidal anions of the stroma (Davson, 1949*b*). It has been claimed by Cogan & Kinsey (1942) that, *in vivo*, the tendency to hydrate is opposed by the osmotic withdrawal of water into the supposedly hypertonic aqueous humour and tears. The theoretical objections to this mechanism have already been put forward (Davson, 1949*a*, *b*; Maurice, 1951), but it was considered desirable to provide experimental evidence that would permit of an unequivocal decision between this mechanism and an obvious alternative, namely that the normal state of hydration of the cornea represents a balance between the colloid-osmotic forces favouring the uptake of fluid and salt, and an active-transport mechanism that drives water and salts against the gradient of electrochemical potential. The cells responsible for this active transport could be either those of the epithelium, of the endothelium, or of both.

To appreciate the significance of the experiments to be described, the following introductory considerations will be of value. (*a*) In the excised eye, any increase in hydration consists essentially in the absorption of aqueous humour (Davson, 1949*b*), and its rate will be determined by the permeability of the endothelium to salts and water, on the one hand, and on the efficacy of the hypothetical active-transport mechanism that opposes this uptake. Cooling the eye will have two opposing effects on such a system; by lowering the permeability of the endothelium it will slow the rate of hydration; on the other hand, by decreasing the rate of metabolism it will impair the efficiency of the active-transport mechanism, thereby favouring hydration. By analogy with other active-transport systems, e.g. the erythrocyte (Harris, 1940), we may expect the effect on metabolism to be the more important. Consequently, on comparing two excised eyes, the one maintained at the normal corneal temperature of about 31°, and the other at 7°, we may expect corneal hydration to be greater at the lower temperature. (*b*) As a corollary to the above, we may expect that when a cornea has become heavily hydrated as a result of maintaining it at 7°, warming it to 31° should inhibit further hydration and actually reverse the process, so that fluid is driven out of the cornea. (*c*) If the epithelium contributes to the active-transport process, removal of this layer may be expected to favour corneal hydration in the excised eye maintained at 31°, but to have a negligible effect at 7°, when metabolism is very small. (*d*) If the endothelium also contributes to the active process, the hydration of a cornea maintained, without epithelium, at 31° may be less than that of a normal cornea maintained at 7°. (*e*) If the physical removal of water (by osmosis into the evaporated tears in the normal intact eye) is an insignificant factor in maintaining normal hydration, then in the excised eye we may expect that variations in the relative humidity of the surroundings will be without significant effects on the corneal hydration. (*f*) Interference with metabolism, for example by removal of oxygen, might be expected to increase hydration.

It will be seen that all these predictions are verified by experiment; they are all inconsistent with the purely physical mechanism mentioned above.

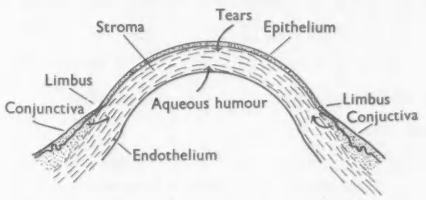

Fig. 1. Illustrating fluid relationships of the cornea; the arrows indicate direction of fluid intake.

METHODS

Eyes, freshly excised from rabbits given a lethal dose of Nembutal (sodium pentobarbitone), were employed throughout this work. They were placed, for ease of handling, on shallow Perspex cups so that the cornea and part of the sclera were exposed to the atmosphere. The cups were placed on a tray in a Perspex box of about 1 l. capacity; the humidity in the box was controlled by passing moist air through it at a rate of about 100 ml./min. The air, before passing through the box, bubbled through two Winchester quart bottles in series, containing 0·9% (w/v) NaCl. The whole system was immersed in a water bath at 31°, the temperature being maintained constant to within about 0·02°. After some 16 hr. the eyes were successively removed and from each the cornea was excised rapidly and blotted, and its water content was determined by weighing before and after drying at 110°. When it was necessary to study pairs of eyes at the same temperature, but under different atmospheric conditions, a Perspex box divided into two compartments was used; each compartment had its own inlet and outlet tube. For maintenance of eyes at 7° it was

not, in general, considered necessary for the purposes of these experiments to control the humidity, so for most of these experiments the eyes were kept in a closed box containing cotton wool moistened with 0·9% NaCl, the box being placed in a refrigerator. Usually, to check on the degree of evaporation that took place during the experimental period, the eyes were weighed before and after their sojourn in the box. The temperature of 7° mentioned throughout this work must be interpreted as the temperature aimed at, rather than the true temperature in any given experiment, since an ordinary commercial refrigerator was employed, the internal temperature of which varied between about 5° and 10°.

RESULTS

The normal hydration. The corneae from ten separate animals were excised immediately after death and their water contents determined. The range of values so obtained extended between 73·7 and 77·5%, with a mean of 75·9. We may note that the percentage of water in a tissue is usually an insensitive index to any changes in its hydration, and it is preferable to indicate the weight of water per unit weight of solid matter; thus the range of hydration indicated above, expressed in this way, is 2·8–3·45 g./g. and the mean is 3·15 g./g. In the following description the hydration will be expressed in both ways whenever convenient.

Eyes maintained at 31 and 7°. The results on four pairs of eyes, each pair from the same animal, are shown in Table 1; in every case the hydration was considerably less in the eye maintained at normal temperature. During the course of this work, eyes from twenty-four separate animals were maintained at 31° for periods ranging from 17 to 23 hr., whilst twelve eyes were maintained at 7°; the average water content of the eyes maintained at 31° was 77·5% ± 0·14 (S.E.) or 3·45 ± 0·04 g./g., whilst that of the eyes maintained at 7° was 82·2% ± 0·3 or 4·6 ± 0·1 g./g. These results show that the cornea of an excised eye, stored at 31°, is less heavily hydrated than one stored at a low temperature. It is interesting, moreover, that of the twenty-four eyes kept at 31° under these conditions, the corneae of twelve had a hydration within the normal range of 73·7–77·5%.

Reversal of hydration. Table 2 shows the effect of warming eyes that had previously been maintained at 7°. Pairs of eyes from the same animals were kept at 7° for 15–18 hr.; the cornea from one eye was excised and its water content determined, whilst the other eye was transferred to the box at 31° for a period of 6–8 hr., when its corneal water content was likewise determined. In every experiment the period at 31° resulted in a decrease in the hydration.

Removal of the epithelium. In these experiments the epithelium was scraped off the cornea of one eye of each pair; both eyes were maintained at the stated temperature for periods ranging from 6 to 19 hr. The results are summarized in Table 3. In all cases the eye without its epithelium showed the greater degree of hydration, but the effect was much more marked at the normal temperature. The average water content of these stripped corneae, maintained at 31°, was 79·4% ± 0·4 (S.E.) or 3·85 ± 0·1 g./g. If we compare this hydration with the average for normal eyes maintained at 7°, namely 82·2% ± 0·3 or 4·6 ± 0·1 g./g., we see that it is quite significantly less. If the endothelium were acting merely as a passive membrane, we should expect the hydration at 31° to be considerably higher than that of a normal eye at 7°; this finding

Table 1. *Comparison of eyes maintained at normal and low corneal temperatures*

Expt. no.	Temp. (°)	Time (hr.)	Water content (g./100 g. tissue)	Water content (g./g. solid)
1	7	15	82·8	4·8
	31	—	77·2	3·4
2	7	15	82·8	4·8
	31	—	77·0	3·35
3	7	17	82·1	4·65
	31	—	78·2	3·6
4	7	7	78·5	3·65
	31	—	77·8	3·5

Table 2. *The effect of subsequent warming on eyes maintained for 15–18 hours at 7°*

Column *A* gives the water content after the period at 7°; column *B* the water content after a further period of 6–8 hr. at 31°.

Expt. no.	Water content (g./g. solid) (*A*)	(*B*)	Change (%)
1	4·35	3·3	24
2	5·1	3·0	41
3	4·45	3·7	17
4	4·65	3·9	16

Table 3. *The effect of removal of the epithelium on the maintainance of corneal hydration*

Temp. (°)	No. of expts.	Average time (hr.)	Water content (g./g. solid) Control	Epithelium removed	Mean difference (Test − Control)
31	7	12·5	3·4	3·85	0·45 ± 0·09 (S.E.)
7	3	14	4·15	4·3	0·15 ± 0·05 (S.E.)

suggests, therefore, that the endothelium is capable of resisting to some extent the tendency of the cornea to absorb aqueous humour.

The effect of evaporative loss. In spite of the precautions taken to minimize losses due to evaporation at 31°, it was only rarely that the eyes, maintained at this temperature for 16 hr. or so, preserved a constant weight; the average loss was some 0·5 % of the total weight of the eye. It might be argued that this was a significant factor in maintaining the approximately normal hydration that was so frequently observed at 31° by comparison with the grossly abnormal hydration at 7°. So far as this comparison is concerned, however, this consideration is groundless, since in all cases where the weights were measured it was found that the loss by evaporation was considerably greater at the lower temperature; moreover, by deliberately increasing the rate of evaporation at 7°, by passing through the box air that had been bubbled through 10 % (w/v) NaCl, the corneal hydration was no different. As a further check on the significance of this factor, pairs of eyes were placed in the two-compartment box at 31°. Through one compartment was passed air that had been bubbled through distilled water (or, in three experiments, through 0·9 % (w/v) NaCl) and through the other air that had been bubbled through 10 % (w/v) NaCl. The results on the corneal hydration are summarized in Table 4, from which it will be seen that, in spite of the quite large difference in evaporative loss, the average hydration was the same under the two conditions, due presumably to the fact that any evaporative loss from the cornea is rapidly compensated by an influx of water from the aqueous humour. It will be noted that the evaporative loss with air bubbled through water or 0·9 % (w/v) NaCl was quite high in these experiments; this was because the compartments in the box employed were much smaller than the box employed for the other experiments. In consequence, the rate of flow of air over the eyes was greater. Although the air was dispersed as fine bubbles by passing through porous stone, it was apparently not completely saturated.

Table 4. *The effect of increasing the rate of evaporative loss from the eye, maintained for* 16 *hours at* 31°, *on the hydration of the cornea*

Mean of seven experiments. Limits are standard errors.

Water content (g./100 g. tissue)	Water content (g./g. solid)	Loss in wt. of whole eye (%)
Air bubbled through distilled water or 0·9 % (w/v) NaCl		
77·4±0·16	3·4±0·03	0·8
Air bubbled through 10 % (w/v) NaCl		
77·6±0·44	3·5±0·09	3·1

Effect of anoxia. Pairs of eyes were placed in the two-compartment box; over one, moist air was passed, over the other, moist N_2. The effect of anoxia was so unequivocal that only two experiments were performed. The results were as follows: Hydration of cornea in air (1) 3·4 g./g.; (2) 3·7 g./g. Hydration of cornea in N_2 (1) 8·1 g./g.; (2) 7·8 g./g. Thus in each case the cornea in N_2 was more than twice as hydrated as that in air.

DISCUSSION

The results described here leave no doubt as to the importance of a normally functioning metabolism in maintaining the normal corneal hydration, and are consistent with the view that the tendency for the cornea to absorb fluid and crystalloids from its surroundings is opposed by an active-transport mechanism. On this basis, then, we may discuss very briefly the salient features of the water economy of the cornea. As indicated earlier, a slow uptake of fluid and crystalloids may be expected from the tears and aqueous humour, its speed being determined by the low permeability of the epithelium and endothelium to salts. In the long run, this uptake will be independent of the tonicities of the tears and aqueous humour, although fluctuations in these values may have temporary effects (von Bahr, 1948). A further uptake is possible in the limbal region, directly from the blood capillaries; but, although this is unimpeded by any selective membranes, its extent will be limited by the relatively large distances through which diffusion must take place. It is necessary, therefore, to postulate a mechanism that will efficiently oppose the entry of both water and crystalloids. Two main possibilities present themselves (Maurice, 1951): first, there may be an active transport of water into the tears and aqueous humour by the epithelium and endothelium respectively; this would lead to a rise in the concentration of crystalloids in the stroma which would then favour their outward diffusion. Alternatively, we may postulate an active extrusion of a crystalloid, e.g. Na^+, which would lead, in a similar way, to a passive removal of water. At present there is no evidence to permit a decision between the alternatives. We may note that the active transport of water, to be efficient, must occur across both the epithelium and endothelium; otherwise the removal of water at one side of the cornea would, to a large extent, be nullified by an osmotic entry at the other, since the diffusion of salt through epithelium or endothelium is slow by comparison with that of water. This is consistent with the findings, described in this paper, indicating that both the epithelium and endothelium oppose the entry of fluid into the cornea of the excised eye maintained at 31°. Variation in the tonicity of the

tears, determined by evaporation, will, if the active transport of water by the epithelium is independent of this factor, be largely self-compensating; an increase in evaporative loss, for example, will be fairly rapidly balanced by osmotic withdrawal from the aqueous humour. Thus the mechanism is consistent with the observation, reported here, that the state of hydration is independent of the atmospheric conditions, at any rate within the limits described. It is also consistent with the common observation that the eye may be maintained closed for days without any obvious increase in thickness, or loss of transparency, of the cornea.

The source of energy for the hypothetical active-transport mechanism would probably be largely the oxidative conversion of glucose and glycogen into carbon dioxide and water (Hermann & Hickman, 1948). We may expect interference with metabolism, by robbing the postulated active-transport mechanism of its energy, to cause an increase in hydration. The present work leaves little doubt that anoxia causes a large increase in the hydration in a period of 16 hr.; similar *in vivo* observations may be found scattered through the literature of this subject (e.g. Langham, 1952), and it would seem that the corneal hazing that results from the wearing of contact lenses is due to an impairment of the oxygen supply to the cornea (Smelser & Ozanics, 1953). It might be argued, however, that the results of anoxia are due to the accumulation of abnormal metabolites which influence the state of the corneal colloids. This raises a point that has so far been ignored, namely whether the post-mortem swelling is really an expression of a normal tendency for the corneal colloids to swell *in vivo*, or whether it is secondary to metabolic interference. In the present paper I have accepted the thesis of Cogan & Kinsey (1942) that the corneal colloids do, indeed, tend to swell *in vivo*, and the purpose of this paper was primarily to test the validity of the purely physical mechanism, advanced by these authors as the mode in which this swelling is prevented. The results quite unequivocally dispose of this hypothesis, but they do not necessarily prove that the cornea *in vivo* has any tendency to swell, or, to put the matter more correctly, that the colloid-osmotic pressure exerted by the stroma is not adequately resisted by the mechanical rigidity of the system. A definite answer to this question cannot yet be given; nevertheless, the results of the several different types of experiment described here, whilst each in itself does not give a conclusive answer, when taken together, do provide strong presumptive evidence for the existence of an active-transport mechanism opposing the entry of fluid into the stroma.

SUMMARY

1. The swelling of the cornea in the excised rabbit eye has been studied with a view to establishing whether, in the living eye, there is an active-transport mechanism that normally prevents the uptake of aqueous humour, tears, and limbal capillary filtrate.

2. The following results are described: (*a*) The corneae of eyes maintained for 16 hr. at 31° are far less heavily hydrated than those maintained at 7°, in fact half of the eyes kept at 31° had a hydration within the normal range. (*b*) Subsequent keeping of eyes at 31°, after maintaining them at 7° for 16 hr., causes a reversal of the hydration that takes place at the lower temperature. (*c*) Removal of the epithelium causes the hydration to increase at 31° but has a very small effect at 7°. The hydration of corneae maintained without epithelium at 31° is less than that of intact corneae at 7°, indicating that the endothelium resists the uptake of fluid. (*d*) The state of hydration is apparently independent of the rate of evaporation from the eye. (*e*) Anoxia causes a very marked increase in hydration.

3. All the results are consistent with the existence of an active-transport mechanism but quite inconsistent with the view that the hydration *in vivo* is prevented by an osmotic withdrawal of fluid into the supposedly hypertonic tears and aqueous humour.

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Studies on Sulphatases

9. THE ARYLSULPHATASES OF MAMMALIAN LIVERS

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Kinetic data for the hydrolysis of potassium *p*-acetylphenyl sulphate by fresh rat liver suspensions suggested that the arylsulphatase activity so observed could be attributed to a single enzyme (Dodgson, Spencer & Thomas, 1953) which was later found to be localized mainly in the microsomes of the liver cell (Dodgson, Spencer & Thomas, 1954*a*). Preliminary attempts to purify the microsome enzyme (unpublished data) were handicapped by its insolubility in various buffers and salt solutions of widely differing pH even after treatment of the liver by acetone-drying, alternate freezing and thawing, or use of the tissue disintegrator (Mickle, 1948). Sulphate and phosphate ions had little effect on the enzyme.

These results contrast sharply with the arylsulphatase pattern reported for ox liver (Roy, 1953*a*, *b*; 1954). Using dipotassium 2-hydroxy-5-nitrophenyl sulphate as the assay substrate, Roy was able to separate two arylsulphatases from the soluble material of acetone-dried ox liver and both enzymes were strongly inhibited by sulphate and phosphate ions. With the same substrate the arylsulphatase activity of mouse liver was found to be mainly concentrated in the mitochondria (Roy, 1953*a*).

The present work, a preliminary report of which has already appeared (Dodgson, Spencer & Thomas, 1954*b*), shows how the occurrence of three different arylsulphatases in mammalian livers enables these contrasting findings to be reconciled.

METHODS

Arylsulphates. Potassium *p*-acetylphenyl sulphate (APS) and potassium *p*-nitrophenyl sulphate (NPS) were prepared by the method of Burkhardt & Lapworth (1926) and dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS) according to Roy (1953*a*).

Measurement of arylsulphatase activity. The method previously used for APS (Dodgson, Lewis & Spencer, 1953) was also adopted for NPS but using the $\lambda_{max.}$ and $\epsilon_{max.}$ determined for *p*-nitrophenol (Dodgson & Spencer, 1953). The use of NPS was restricted to liver extracts, as recoveries of *p*-nitrophenol from fresh liver suspensions are low (Dodgson & Spencer, 1953). The faint turbidity of the final solutions was the same in both test and controls and duplicate determinations were satisfactory. Turbidity only interfered, by raising control spectrophotometer readings to a high base level when very concentrated liver suspensions and extracts were used and in such cases the original ethanol precipitation method (Dodgson, Spencer & Thomas, 1953) was adhered to. The method of Dodgson, Melville, Spencer & Williams (1954) was used when NCS was the substrate. Although fresh liver suspensions can reduce liberated 4-nitrocatechol (Dodgson & Spencer, 1953), the high activity of rat liver against nitrocatecholsulphate allowed the use of liver suspensions and fractions separated in isotonic sucrose at concentrations at which 4-nitrocatechol could be quantitatively recovered over an incubation period of 1 hr.

In all enzyme assays sodium acetate–acetic acid was the buffer used at a final concentration in the incubation mixture of 0·5 M. The buffer, substrate and enzyme solutions were adjusted independently to the required pH with acetic acid or NaOH before mixing. Incubation was for 1 hr. at 37·5° in all cases and the concentration of enzyme solution was such that the spectrophotometric readings corresponded to an extinction E ($\log_{10} I_0/I$) of less than 1.

RESULTS

The experimental methods used by Roy (1953*a*) differed from those of Dodgson, Spencer & Thomas (1953) in three main respects, namely, animal species enzyme substrate and the use of acetone-dried preparations of ox liver as opposed to fresh suspensions of rat liver. The significance of each of these differences was examined in relation to the contrasting findings of the two groups of workers.

The effect of acetone. Most of the known facts could be explained by assuming that the two enzymes separated from ox liver were artifacts due to the acetone treatment (Roy, 1953*a*), whereas in the fresh rat liver only one enzyme was present. However, the kinetic behaviour of rat liver arylsulphatase towards APS was not affected by acetone treatment, suspensions of whole acetone-dried rat liver showing substrate concentration (Fig. 1) and pH/activity curves which were the same as those previously obtained with fresh liver (Dodgson, Spencer & Thomas, 1953). Neither the curves for fresh rat liver nor those for acetone-dried rat liver indicated the presence of more than one arylsulphatase. Acetone treatment was not therefore responsible for the difference between rat and ox arylsulphatases. This conclusion was subsequently

strengthened by the demonstration of the presence of the same two sulphatases in both fresh ox liver and acetone-dried preparations (Roy, 1954).

Species difference. Substrate concentration/activity curves for suspensions of acetone-dried liver of the rat, ox, mouse and rabbit (Fig. 1) with APS as the substrate showed a species difference. Whereas the rat and rabbit enzymes gave smooth Michaelis curves, the rabbit showing marked inhibition with excess substrate, ox and mouse livers gave anomalous discontinuous curves similar to those found by Roy (1953*a*) for the soluble material of acetone-dried ox liver when NCS was the substrate. The curves for ox and mouse could be interpreted as showing the presence of two arylsulphatases.

Substrate difference. To confirm this apparent species difference the substrate concentration/activity curves were repeated using NCS at pH 6·0. The smooth curves obtained with the whole acetone-dried livers of the four animal species (Fig. 2) were of the normal pattern and gave straight line graphs when $1/v$ was plotted against $1/S$ (Lineweaver & Burk, 1934) except in the cases where inhibition by excess substrate occurred. The experiment was repeated at pH 5·3, the pH at which Roy (1953*a*) had obtained an anomalous curve with ox liver, but the curves obtained were quite smooth. Since these findings differed from those obtained with APS, the curves were repeated with a third substrate, NPS. With this substrate the acetone-dried livers of all four species gave anomalous Michaelis curves (Fig. 3) which suggested the presence of two arylsulphatases in each case, and that the species difference observed when APS was the substrate was only apparent. The findings with APS and NCS emphasize that Michaelis curves of normal type can readily be given by mixtures of two 'isodynamic' enzymes. Whereas discontinuous curves may be safely interpreted as showing the presence of more than one enzyme, the normal type of curves may sometimes be ambiguous. The choice of the rat as the animal species and APS as the substrate in earlier work (Dodgson, Spencer & Thomas 1953) was unfortunate in that no indication of the presence of more than one arylsulphatase was given; the use of other species or substrate would have revealed the complex nature of mammalian arylsulphatase.

Fractionation of acetone-dried liver. The sulphatases measured by Roy (1953*a*) with NCS could be extracted from acetone-dried ox liver with

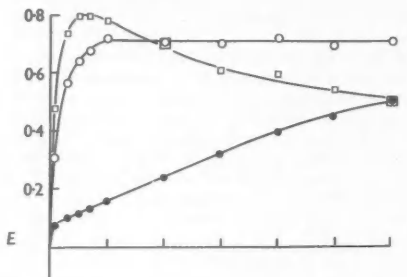

Fig. 1. Substrate concentration/activity curves for the suspensions of acetone-dried liver of rat, ox, mouse and rabbit and for the residue and supernatant obtained by extraction of the acetone-dried rat liver with water for 1 hr. at 37·5°. The substrate was potassium *p*-acetylphenyl sulphate in 0·5M sodium acetate, pH 7·2, and incubation was for 1 hr. at 37·5°. The curves are not quantitatively related to each other. ○—○, rat; ×—×, ox; ■—■, mouse; △—△, rabbit; □—□, residue after extraction of acetone-dried rat liver with water; ●—●, supernatant after extraction of acetone-dried rat liver with water.

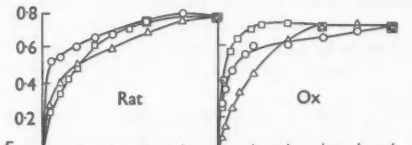

Fig. 2. Substrate concentration/activity curves for the suspensions of acetone-dried liver of rat, ox, mouse and rabbit and for the residue and supernatant obtained by extraction of the acetone-dried livers with water for 1 hr. at 37·5°. The substrate was dipotassium 2-hydroxy-5-nitrophenyl sulphate in 0·5M sodium acetate, pH 6·0, and incubation was for 1 hr. at 37·5°. The curves are not quantitatively related to each other. □—□, whole acetone-dried liver; ○—○, residue after extraction of acetone-dried liver with water; △—△, supernatant after extraction of acetone-dried liver with water.

Table 1. *Fractionation of acetone-dried rat liver*

The activity of each fraction arising from 1 g. of acetone-dried liver was measured with APS 0·007 M, pH 7·2, NPS 0·006 M, pH 7·0 and NCS 0·04 M, pH 6·0.

	Suspension of whole acetone powder			Insoluble fraction			Soluble fraction			Recovery (insoluble + soluble fraction)		
	APS	NPS	NCS	APS	NPS	NCS	APS	NPS	NCS	APS	NPS	NCS
Activity (μg. phenol liberated)	9180	6870	149500	8170	5670	13200	222	180	137500	8392	5850	150700
% of activity of whole powder	100	100	100	89·0	82·4	8·91	2·4	2·6	92·1	91·4	84·6	101·0
Ratio of activities (units/mol.wt.)	1·38 : 1 :		19·5	1·47 : 1 :		2·09	1·26 : 1 :		685	—	—	—

water, whereas similar treatment of either ox or rat liver failed to bring more than 3% of the arylsulphatase activity into solution when APS (0·007 M, pH 7·2) was the substrate (Table 1). This suggested that a preliminary separation of the enzymes of rat liver could be achieved by dividing the acetone-dried liver into soluble and insoluble fractions.

Acetone-dried rat liver (0·1 g.) was incubated for 1 hr. at 37·5° with 10 ml. water and then centrifuged at 20000 *g* for 30 min. at 0°. The residue was washed twice with water (5 ml.), recentrifuged, the supernatant and washings were combined and the remaining residue was resuspended in water. The activities of the whole acetone-dried liver suspension, the debris and the supernatant were measured in the presence of 0·5 M acetate against APS (0·007 M, pH 7·2), NPS (0·006 M, pH 7·0) and NCS (0·04 M, pH 6·0). The conditions for APS and NCS were those found to be optimum for the whole powder (Figs. 1, 2), and those for NPS being the substrate concentration at which the Michaelis curve for the whole powder showed a discontinuity (Fig. 3).

The results show that 92% of the activity against NCS appeared in the soluble material, while less than 3% of the activity against APS and NPS was in this fraction. Furthermore, repetition of the fractionation procedure on the debris resulted in the extraction of more NCS activity without removing an equivalent amount of APS activity (Table 2). Similar findings were made when 0·5 M acetate, pH 6·8, replaced water as the extracting medium.

Substrate concentration/activity curves for APS, NPS and NCS (Figs. 1–3), using the soluble and insoluble fractions of rat liver, prepared as described above, showed that separation of the arylsulphatases had been achieved. With APS and NPS the insoluble material had optimum substrate concentration similar to those found for the whole acetone-dried rat liver (Figs. 1, 3), and fresh liver suspensions (Dodgson, Spencer & Thomas, 1953). The soluble fraction had only about 3% of the activity of the insoluble fraction at the optimum substrate concentration for the latter, but showed

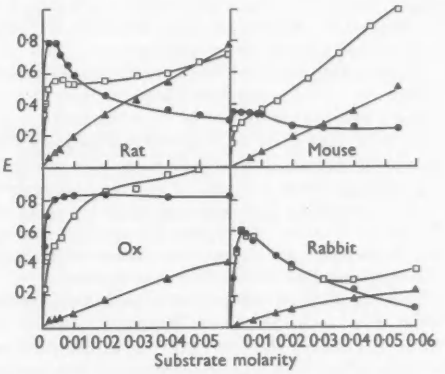

Fig. 3. Substrate concentration/activity curves for the acetone-dried liver of rat, ox, mouse and rabbit and for the residue and supernatant obtained by extraction of the acetone-dried livers with water for 1 hr. at 37·5°. The substrate was potassium *p*-nitrophenyl sulphate in 0·5 M sodium acetate, pH 7·0, and incubation was for 1 hr. at 37·5°. The curves are not quantitatively related to each other. □—□, whole acetone-dried liver; ●—●, residue after extraction of acetone-dried liver with water; ▲—▲, supernatant after extraction of acetone-dried liver with water.

Table 2. *Fractionation of once-extracted acetone-dried rat liver*

Acetone-dried rat liver which had been extracted once with water for 1 hr. at 37·5° was dried with acetone and then re-extracted with water. The activity of the soluble and insoluble fractions towards APS (0·007 M, pH 7·2) and NCS (0·04 M, pH 6·0) are quoted as % of the activity of the once-extracted material.

Fraction	Activity	
	APS	NCS
Whole powder (once-extracted)	100	100
Insoluble fraction	95·7	84·6
Soluble fraction	1·8	16·2
Recovery	97·5	100·8

considerable activity at higher substrate concentrations. The optimum substrate concentration of the soluble material with APS and NPS was shown, in a separate experiment, to be greater than 0·175 M, but the curves could not be carried to higher concentrations owing to the insolubility of the substrates. The NCS curves for soluble and insoluble fractions showed little difference when measured at pH 6·0.

These results indicate that at least two enzymes are present in acetone-dried rat liver. The 'insoluble' enzyme has high affinity for APS and NPS, whereas the 'soluble' enzyme has a low affinity for these substrates. On the other hand, the bulk of the activity towards NCS is associated with the soluble rather than the insoluble fraction (Table 1), although the affinities of these fractions for this substrate do not appear to differ greatly.

Work on the inhibition of the arylsulphatases with phosphate ions described in the latter part of this paper suggests that under the experimental conditions used most of the NCS activity of the insoluble portion is actually due to residual soluble enzyme.

Similar patterns existed for the ox, mouse and rabbit livers (Figs. 2, 3), although from the shape of the curves for the whole acetone powders it may be inferred that the proportion of 'insoluble' and 'soluble' enzymes differs with each species.

Intracellular distribution of rat liver arylsulphatase. With APS as the substrate, Dodgson, Spencer & Thomas (1954*a*) showed that the arylsulphatase activity of rat liver was predominantly localized in the microsomes of the liver cell, while in the mouse 70 % of the activity against NCS was present in the mitochondria (Roy, 1953*a*). It was probable that the use of different substrates rather than a species difference was responsible for these contrasting findings, since the localization of the arylsulphatase activity of rat liver was similar to that of the mouse when NCS was the assay substrate.

The components of rat liver cells were fractionated by the isotonic sucrose technique (Schneider, 1948) and the fractions simultaneously assayed with NCS (0·04 M in 0·5 M acetate, pH 6·0) and APS (Dodgson, Spencer & Thomas, 1953). Sucrose (0·025 M) had no effect on the arylsulphatase activity when either NCS or APS were the substrates. The figures for APS activity (Table 3) were substantially the same as those previously recorded (Dodgson, Spencer & Thomas, 1954*a*) and showed that the greatest activity was present in the microsomes whilst the mitochondria and final supernatant were only weakly active. With NCS the mitochondria were predominantly active although both the microsomes and the final supernatant fractions retained considerable activity (Table 3).

From Fig. 1 and Table 1 it would appear that assay with APS (0·007 M at pH 7·2) measures only the 'insoluble' enzyme, and that the activity of and competition by the 'soluble' enzyme is very small. On the other hand, the 'soluble' enzyme is responsible for the bulk of the activity measured when NCS (0·04 M, pH 6·0) is the substrate (cf. Fig. 2 and Table 1).

The 'insoluble' enzyme is therefore localized in the microsomes and the 'soluble' enzyme in the mitochondria and to a lesser extent in the microsomes and final supernatant fraction. Confirmation of this was obtained by preparing substrate concentration/activity curves with APS (Fig. 4) for the

Table 3. *The localization of the arylsulphatase activity of the rat liver cell*

The fractions separated by the method of Schneider (1948) were assayed against APS (0·007 M, pH 7·2) and NCS (0·04 M, pH 6·0). The results are expressed as a percentage of the activity of the initial whole tissue suspension and are the average values obtained from four different experiments.

Fraction	Activity (% of initial whole tissue suspension) APS	NCS
Nuclear	12·5	9·1
Mitochondrial	6·0	62·0
Microsomal	61·7	21·5
Supernatant	2·6	16·6
Recovery	82·8	109·2

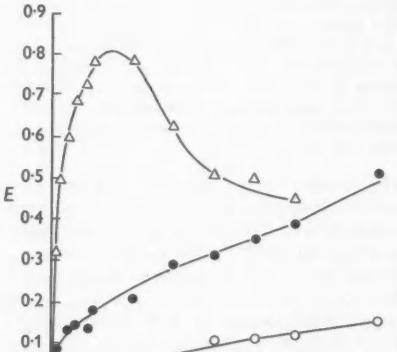

Fig. 4. Substrate concentration/activity curves for the mitochondrial, microsomal and supernatant fractions obtained by differential centrifuging of the cellular components of rat liver in isotonic sucrose solution according to the method of Schneider (1948). The substrate was potassium *p*-acetylphenyl sulphate in 0·5 M sodium acetate, pH 7·2, and incubation was for 1 hr. at 37·5°. The curves are not quantitatively related to each other. ●—●, mitochondrial fraction; △—△, microsomal fraction; ○—○, supernatant fraction.

fractions isolated using isotonic sucrose (Schneider, 1948) when it was seen that the microsomal fraction gave a curve similar to that of the insoluble material of acetone-dried liver (Fig. 1) and that both the mitochondria and the final supernatant gave curves similar to that of the 'soluble' enzyme. These curves show that the 'soluble' and 'insoluble' enzymes do exist in fresh liver and are not just artifacts arising from acetone treatment. The NCS curves for the mitochondria microsomes and final supernatant were almost identical and similar to those shown in Fig. 2.

The complex nature of the 'soluble' enzyme

The substrate concentration/activity curves with APS, NPS and NCS for the soluble fraction of acetone-dried rat liver (Figs. 1–3) gave no indication of the presence of more than one enzyme. However, as previously explained, such curves may be given by mixtures of enzymes. In view of Roy's claim (1953*a*, *b*; 1954) to have separated two sulphatases from the soluble material of acetone-dried ox liver, attempts were made to detect the presence of more than one 'soluble' arylsulphatase in rat liver.

Fractionation of rat liver by the method of Roy (1953*b*, 1954) to the stages *A*-2 and *B*-2 gave fractions which showed substrate concentration/activity curves at pH 4·75 and 5·75 which were not significantly different. However, a satisfactory demonstration of the presence of two enzymes was obtained by a simple fractionation of acetone-dried rat liver.

A 10% (w/v) suspension of acetone-dried rat liver in water was incubated at 37·5° for 1 hr. and then centrifuged at 20000 *g*. The clear supernatant was fractionated with acetone between the limits 0–30 and 50–60% acetone (v/v) observing the precautions detailed by Askonas (1951). The precipitates were dried with acetone under ice-cold conditions. The fractions were suspended in water, clarified by centrifuging at 0° and re-fractionated between the limits 0–30 (fraction I), and 50–60% (fraction II) acetone, respectively. The precipitates were dissolved in water, dialysed against running water for 36 hr., clarified by centrifuging and kept in the frozen state.

The NPS and NCS substrate concentration/activity and pH/activity curves were constructed for fractions I and II (Figs. 5, 6). The fractions showed obvious differences, particularly for the NCS substrate and pH curves, although with NPS the difference was less marked in the case of the pH curve and not apparent in the substrate curves. In no case was the difference as great as that found for the two soluble enzymes of ox liver (Roy, 1953*a*). The difference in kinetic behaviour between the fractions does, however, suggest that at least two enzymes are present in the soluble material of acetone-dried rat liver. The great difference between the curves for fractions I and II and similar curves for the insoluble material of acetone-dried rat liver (Figs. 5, 6) makes it improbable that either of the two enzymes of the soluble material is identical with the main constituent of the insoluble material. This was confirmed by the effect of phosphate ions on the activities of the various

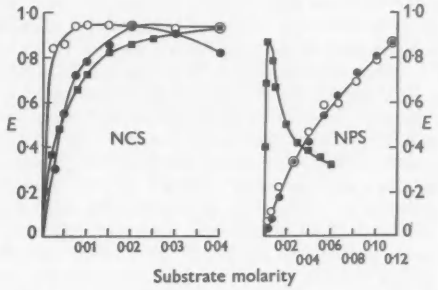

Fig. 5. Substrate concentration/activity curves for fractions of rat liver showing the presence of three different arylsulphatases. The 'insoluble' enzyme fraction was the residue from acetone-dried rat liver after repeated extraction with water, while enzyme fractions I and II were prepared as described in the text (p. 33). The substrates used were dipotassium 2-hydroxy-5-nitrophenyl sulphate (in 0·5 M sodium acetate, pH 5·75) and potassium *p*-nitrophenyl sulphate (in 0·5 M sodium acetate, pH 6·5). ●—●, fraction I enzyme; ○—○, fraction II enzyme; ■—■, 'insoluble' enzyme.

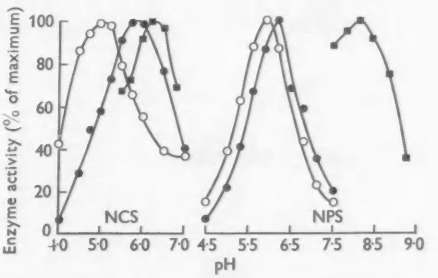

Fig. 6. pH/Activity curves for three fractions of rat liver showing the presence of three different arylsulphatases. The 'insoluble' enzyme fraction was the residue from acetone-dried rat liver after repeated extraction with water, while enzyme fractions I and II were prepared as described in the text (p. 33). The substrates used for fractions I and II were dipotassium 2-hydroxy-5-nitrophenyl sulphate (0·02 M) and potassium *p*-nitrophenyl sulphate (0·05 M). The insoluble enzyme was measured using 0·04 M dipotassium 2-hydroxy-5-nitrophenyl sulphate and 0·0025 M potassium *p*-nitrophenyl sulphate. In each case enzyme activity was measured in the presence of 0·5 M sodium acetate–acetic acid buffer adjusted to the required pH. ●—●, fraction I enzyme; ○—○, fraction II enzyme; ■—■, 'insoluble' enzyme.

fractions. The degree of inhibition was determined using the optimum conditions of substrate concentration and pH (Figs. 5, 6) except when NPS was the substrate for fractions I and II, in which case a substrate concentration of 0·05 M was used. The arylsulphatase activity of fractions I and II was completely inhibited by 0·025 M phosphate when either NPS or NCS was the substrate, but the insoluble material obtained from acetone-dried liver was only slightly inhibited (approx. 10 %) by the same concentration of phosphate when NPS and APS (0·007 M, pH 7·2) were the substrates.

When NCS was the substrate the activity of the insoluble material was inhibited 96 % by 0·025 M phosphate. This result may be interpreted by assuming that the 'insoluble' enzyme is only slightly inhibited by phosphate, as shown with APS and NCS, and that the activity measured with NCS under the conditions stated is actually due to residual soluble enzymes which are strongly inhibited by phosphate. Table 2 shows that a considerable amount of soluble enzyme remains in once-extracted acetone-dried liver.

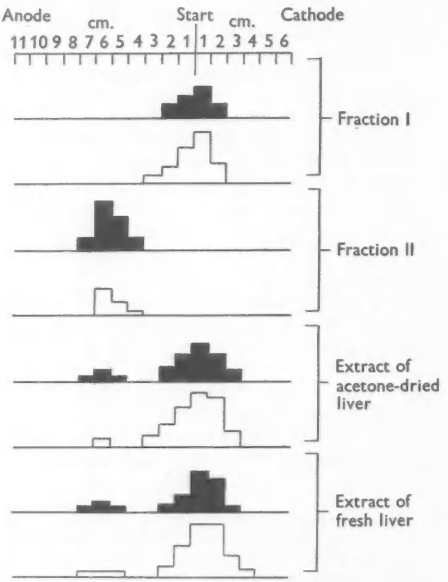

Fig. 7. Electrophoretic movement of the arylsulphatases of fractions I and II and the aqueous extracts of acetone-dried and fresh rat liver. Enzyme activity was assayed with dipotassium 2-hydroxy-5-nitrophenyl sulphate using two different sets of experimental conditions (0·02 M, pH 6·0, open figures; 0·008 M, pH 4·8, closed figures). The activities for the two sets of conditions are approximately related to each other within each fraction only. Electrophoresis in veronal–acetate–HCl buffer, pH 7·1, was for 16 hr. at 200 v using Whatman no. 100 paper.

Paper electrophoresis. Confirmation of the presence of two separate enzymes in the soluble material of rat liver was obtained by paper electrophoresis. The soluble material was extracted by incubating 20 % (w/v) water suspensions of fresh liver and 10 % (w/v) suspensions of acetone-dried liver for 1 hr. at 37·5° and centrifuging at 20000 *g* for 1 hr. Paper electrophoresis of these extracts and fractions I and II was carried out by the technique described by Roy (1954), except that each paper was divided longitudinally after electrophoresis and 1 × 1·5 cm. strips of each half were assayed over 30 min. with either 0·008 M NCS, pH 4·8 or 0·02 M NCS, pH 6·0. These assay conditions were near optimum for fractions I and II respectively (Figs. 5, 6). Fractions I and II appeared to be substantially free from contamination by each other even when the assay conditions were those optimum for the suspected contaminant (Fig. 7). Fraction I is comparable to sulphatase *B* of ox liver (Roy, 1954), since it moved towards the anode and its kinetic behaviour followed more closely that of ox sulphatase *B* than did that of fraction II. Fraction II showed similar points of comparison with ox sulphatase *A*.

Using either set of assay conditions, the extracts of fresh and acetone-dried liver showed two separate bands of arylsulphatase activity which moved at the same speeds as the enzymes of fractions I and II (Fig. 7). The activity of the bands with the same mobility as fraction I was greatest under the conditions which were optimum for this fraction and a similar relationship existed for the other bands and fraction II. The fraction II enzyme appeared to be present in the liver in small amounts only and the ratio of activity of the two bands was of the order of 1:100 under the respective optimum conditions (cf. Roy, 1954). When the paper strips were assayed over 2 hr. some activity appeared between the two main spots, suggesting that some tailing off of the enzyme bands had occurred. The demonstration of two 'soluble' enzymes in fresh liver confirms Roy's findings (Roy, 1954) that they are not artifacts due to acetone treatment.

DISCUSSION

The arylsulphatase activity of rat and ox livers can be attributed to a complex system of at least three arylsulphatases which possess widely different substrate affinities. Fractionation of rat liver in isotonic sucrose solution showed that two of the enzymes ('soluble') were mainly associated with the mitochondria and could be obtained in solution by techniques which rupture these granules, such as acetone-drying and alternate freezing and thawing.

Table 4. *Conditions used by previous workers for the study of mammalian arylsulphatases*

From an examination of these conditions the particular enzyme studied in each case has been inferred and included in the table under the heading 'enzyme studied'. Insufficient details are given by Abbott & East (1949) to enable a decision to be given. The pH and substrate concentrations shown are either those found to be optimum for the enzyme under consideration or those used for assay purposes.

Animal	Organ	Method of preparation of enzyme	Substrate	Substrate concentration (M)	pH	Enzyme studied	Reference
Pig	Kidney	Washed acetone-dried tissue	Potassium phenyl sulphate	0·047	$CaCO_3$	'Insoluble'	Hommerberg, 1931
Rabbit	Liver	Sucrose and glycerine extracts	NPS	0·0005	5·9	'Insoluble'	Morimoto, 1937
Rabbit	Liver	Glycerine extract	NPS	0·0005	6·1	'Insoluble'	Tanaka, 1938
Rat	Liver and other tissues	Centrifuged water extracts	NPS	0·001	5·8	'Insoluble'	Huggins & Smith, 1947
Rat	Liver and other tissues	?	NPS	?	6·6	?	Abbot & East, 1949
Rat	Liver and other tissues	Extracts (?)	NCS	0·0025	6·0	'Soluble'	Robinson, Smith & Williams, 1951
Rat, ox, rabbit and pig	Adrenals	Whole tissue	NPS	0·001	5·8	'Insoluble'	Malström & Glick, 1952
Rat	Liver and other tissues	Whole tissue	APS	0·007	7·2	'Insoluble'	Dodgson, Spencer & Thomas, 1953*b*, 1954*a*
Ox	Liver	Centrifuged extract of acetone-dried liver	NCS	0·003 >0·06	4·9 5·7	'Soluble' 'Soluble'	Roy, 1953*a*, *b*, 1954
Rabbit	Liver and kidney	Centrifuged NaCl extract	NPS	>0·15	6·02	'Soluble'	Maengwyn-Davies & Friedenwald, 1954

It was not possible to attribute the appreciable activity of the final supernatant after fractionation to the cytoplasmic fluid of the unbroken cell since it might possibly have been extracted from the mitochondria during the fractionation procedure. The remaining enzyme ('insoluble') occurs predominantly in the microsomes and is only present in negligible amounts in the mitochondria and final supernatant. Techniques used for disrupting mitochondria fail to bring this enzyme into solution, and the only known method of achieving this is by the use of surface-active agents (unpublished data). The complex nature of liver arylsulphatase is not confined to the ox and rat, since mouse, rabbit and human (unpublished) livers have been shown to contain both 'soluble' and 'insoluble' enzymes. No investigation has yet been made into the homogeneity of the 'soluble' enzymes of these species.

It is now obvious that in previous work Dodgson, Spencer & Thomas (1953, 1954*a*) have measured only the 'insoluble' enzyme, since at the concentration at which APS was used the 'soluble' enzymes have only about 2% of the activity of the 'insoluble' enzyme (Table 1). On the other hand, the work of Roy (1953*a*, *b*; 1954) has been confined to the 'soluble' enzyme since, by discarding the insoluble material of acetone-dried ox liver after extraction with water, all the 'insoluble' enzyme was effectively removed from his preparations. The contrasting findings of the two groups of workers can be adequately explained on this basis. It is pertinent to consider how the work of previous authors is affected by these findings. By comparing experimental conditions used in the past (Table 4) with the curves for the 'soluble' and 'insoluble' enzymes (Figs. 1–3) it appears that workers have mainly measured one or other of these enzymes only. In other cases the method of preparation of the enzyme itself has ensured that one of the enzymes has been discarded before estimation. Thus, Hommerberg (1931) found that the enzyme of pig's kidney could not be brought into solution, and from the conditions of substrate concentration and pH used it is obvious that he was concerned with the 'insoluble' enzyme. Morimoto (1937) and Tanaka (1938), using conditions that measured mainly the 'insoluble' arylsulphatase of rabbit liver, found that the enzyme could be obtained in solution, since centrifuged sucrose and glycerol extracts of the livers were active. However, it is probable that the enzyme was not actually in solution, since the high specific gravity of the extracts would ensure that the microsomes, containing the 'insoluble' enzyme, were not removed under the normal conditions of centrifuging. A similar comment is applicable to the centrifuged extracts of rat tissues made by Huggins & Smith (1947) particularly since at the concentration of NPS used for assay only the 'insoluble' enzyme would be measured. On the other hand, centrifuging of sodium chloride extracts of rabbit liver at 16 000 rev. min.$^{-1}$ for 1 hr. (Maengwyn-Davies & Friedenwald, 1954) effectively removed all the microsomes, and the supernatant solution showed a Michaelis curve similar to that of the 'soluble' enzyme in Fig. 3. The particular enzyme studied by previous authors has been inferred from the conditions used, and a list is presented in Table 4.

The precise assay of the individual arylsulphatases of mammalian tissues presents some difficulties. Although it may be possible to choose specific substrates and conditions which will assay one of the enzymes preferentially, competition by the other two enzymes might cause some interference. The inhibition of the 'soluble' enzymes by the varying amounts of endogenous phosphate ions is a further complicating factor. However, these factors are at a minimum in the case of the assay of rat tissues with APS (Dodgson, Spencer & Thomas, 1953). Under the conditions used (0·007 M, pH 7·2) the activity of and competition by the 'soluble' enzymes is slight (Table 1) and the 'insoluble' enzyme which is being measured is hardly affected by phosphate ions at the concentration found in tissues. The results previously recorded for the distribution of arylsulphatase activity in rat tissues (Dodgson, Spencer & Thomas, 1953) and the localization in the rat liver cell (Dodgson, Spencer & Thomas, 1954*a*) may therefore be considered as descriptive of the 'insoluble' (microsomal) enzyme only.

There is as yet no evidence to suggest that the arylsulphatase activity of sources other than mammalian tissues is of a complex nature. The arylsulphatase of *Alcaligenes metalcaligenes* (Dodgson, Melville, Spencer & Williams, 1954) has been shown to be electrophoretically homogeneous (unpublished data), but in the other cases no such criterion is available and their homogeneity must remain in doubt pending further investigation. Indeed, the flat pH optima exhibited by taka-diastase are suggestive of the presence of more than one enzyme (Robinson, Smith, Spencer & Williams, 1952).

SUMMARY

1. The presence of two arylsulphatases in the livers of rat, ox, rabbit and mouse has been shown. The enzymes can be distinguished by the ready solubility of one of them ('soluble') after disruption of the mitochondria by acetone-drying, incubation in hyper- and hypo-tonic solutions or alternate freezing and thawing.

2. In the rat the 'insoluble' enzyme is localized in the microsomes of the liver cell and the 'soluble' enzyme is found mainly in the mitochondria. The

'insoluble' enzyme has a high affinity for *p*-nitrophenyl and *p*-acetylphenyl sulphates whilst the 'soluble' enzyme has a low affinity for these substrates but a high activity towards nitrocatechol sulphate.

3. The 'soluble' enzyme of rat liver has been further separated into two enzymes which are comparable to sulphatases *A* and *B* of ox liver (Roy, 1954).

4. The work of previous authors was discussed in the light of these findings.

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Fate of Porphobilinogen, Administered Enterally or Parenterally, in the Rat

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Porphobilinogen is excreted in the urine in large quantities in acute porphyria and is always found in the livers of fatal cases of this disease. In experimental porphyria in animals induced by sedormid (allyl*iso*propylacetylurea) (Schmid & Schwartz, 1952) or allyl*iso*propylacetamide (Goldberg, 1953), porphobilinogen is likewise found in the urine and liver. Previous reports on the behaviour of this substance when administered to experimental animals are conflicting, probably because the porphobilinogen used was only partially purified or was available in amounts too small for adequate investigation. Thus Waldenström & Wendt (1939) injected partially purified porphobilinogen into rabbits and found it to be excreted in the urine, while Prunty (1945) failed to find any in the urine under similar conditions. Goldberg, Paton & Thompson (1954) injected porphobilinogen, which had been isolated by Westall (1952), intravenously into one rabbit with an external bile fistula and found traces of uroporphyrin in the urine and a slight increase in the excretion of bile porphyrin.

The following experiments with porphobilinogen were done in order to clarify its mode of excretion in an experimental animal. It is suggested that the results of these studies in the rat may be relevant to human acute porphyria. It was found that porphobilinogen, when administered parenterally to the rat, is excreted into the urine mainly by glomerular filtration. If it is given enterally or parenterally, it is not found in the livers of rats subsequently killed, at a time when the plasma still contains porphobilinogen. These findings suggest that the porphobilinogen, found in the livers of rats with experimental porphyria, has been formed there and has not been transported to the liver from an extrahepatic site.

Falk, Dresel & Rimington (1953) showed that porphobilinogen behaved as a precursor of certain porphyrins in a haemolysed chicken-erythrocyte

system. In the present work, a small but significant conversion of porphobilinogen into coproporphyrin III and uroporphyrin III has been detected in the rat. A brief preliminary communication concerning this work has appeared (Goldberg & Rimington, 1954*a*).

METHODS

General

The Wistar-strain rats used weighed between 150 and 214 g. with the exception of rat no. 7 which weighed 112 g. Rats nos. 9 and 13 were female. They were placed in metabolism cages. In studies lasting 2 weeks, separate daily urinary and faecal specimens were analysed every second day for porphyrins, the specimens not determined on the day of excretion being stored at 2°. In experiments where urine was collected at frequent intervals, e.g. half-hourly or hourly, micturition was induced by a 'tail-pulling' procedure similar to that described by Hutschenreuter (1933). In collecting urine from metabolism cages, the cage was washed with water, until the washings were free from porphobilinogen (see below). In the experiment on rat no. 9, where hourly urine specimens were taken for 20 hr., the interior of the metabolism cage was washed 4 times at the end of each hr. with a total vol. (each hr.) of 200 ml. water.

The porphobilinogen used had been isolated in the crystalline form (Westall, 1952; Cookson & Rimington, 1954) and was administered as the hydrochloride in 0·9% (w/v) NaCl, using 0·5 ml. for parenteral injection and 1 ml. for enteral administration. Intravenous injections were given into a tail vein, subcutaneous into the dorsal area of the rat. All enteral administration was carried out by gastric intubation as described by Goldberg & Rimington (1954*b*).

Haematological values were determined as described (Goldberg, 1954).

Isolation and determination of porphyrins

Urine. Coproporphyrin was extracted by the method of Schwartz, Zieve & Watson (1951). Uroporphyrin from the same specimen of urine was obtained by combining the aqueous and sodium acetate washings from the coproporphyrin extraction, and bringing these to pH 3·0–3·2. The washings were then shaken with ethyl acetate, the aqueous layer was discarded and uroporphyrin extracted with 0·6 N-HCl.

Faeces. Coproporphyrin and protoporphyrin were extracted by the method of Schwartz & Wikoff (1952) with the following modifications. The ethyl acetate was shaken with 20–30 ml. 0·005% aqueous I_2 immediately after extraction of porphyrins with 3 N-HCl. The aqueous layer was discarded and a further extraction with 3 N-HCl carried out, in order to obtain any porphyrin precursor. This was not found in any specimen. The final coproporphyrin extraction in 0·1 N-HCl was not shaken with $CHCl_3$. Uroporphyrin was obtained from the sodium acetate washings of the ethyl acetate layer during the above procedure. These acetate washings were dealt with in the same way as described for urinary uroporphyrin.

The porphyrins, so extracted from urine and faeces, were determined by the method of Rimington & Sveinsson (1950) using a Beckman spectrophotometer, model D.U.

Paper chromatography of porphyrins. This was carried out as described (Goldberg, 1954).

Isolation and determination of porphobilinogen

Urine and tissues. Porphobilinogen was determined as described (Goldberg, 1954). It was assumed that 1 mg. of porphobilinogen is equivalent to 130 Vahlquist (1939) units (Westall, 1952).

Faeces. To a weighed amount of faeces in a centrifuge tube, sufficient water was added to give a thin paste. Clarification was effected by adding 10% (w/v) lead acetate in 3% (w/v) acetic acid, in a volume one-tenth of that of the diluted faeces, mixing and then centrifuging. The supernatant was decanted and excess Pb^{2+} was precipitated with a solution containing NaH_2PO_4 (123 g./l.) and K_2HPO_4 (138 g./l.). The mixture was centrifuged and the supernatant analysed for porphobilinogen as described for urine.

Plasma. Protein was precipitated by adding 0·33 vol. of 20% (w/v) trichloroacetic acid to centrifuged plasma as obtained *post mortem*. The mixture was then centrifuged and the supernatant tested for porphobilinogen. In the living rat 0·1 ml. of blood from the tail was sucked into a 0·1 ml. pipette, previously rinsed with heparin. The blood was discharged into 1·5 ml. of 5% (w/v) trichloroacetic acid contained in a centrifuge tube. This was thoroughly shaken, centrifuged and the supernatant tested for porphobilinogen. The observation that porphobilinogen does not enter erythrocytes (Goldberg & Rimington, 1954*b*) was confirmed in the present study. The blood haematocrit for the rat was taken to be 40 (Wintrobe, 1951) and plasma porphobilinogen was calculated on this basis.

Paper chromatography. This was done as described by Westall (1952) and Cookson & Rimington (1953).

RESULTS

Intravenous injection of porphobilinogen

Excretion pattern of porphobilinogen and porphyrins. Rats nos. 1 and 2 were given intravenous injections of 9·04 and 9·4 mg., respectively, of porphobilinogen after a preliminary period of 8 and 7 days, respectively, during which daily faecal and urinary porphyrins were determined. These determinations were continued during the succeeding 8 days. Porphobilinogen was rapidly excreted in the urine of both rats. It appeared in the urine within 10 min. of injection and was detectable until 8 hr. later. In rat no. 1 specimens of urine were collected every 2 hr. during the 12 hr. period after injection. The results of porphobilinogen, uroporphyrin and coproporphyrin determinations on these specimens are illustrated in Fig. 1. This shows that nearly half of the total porphobilinogen excreted in the urine (6·07 mg.) was passed in the first 2 hr. after injection, while the excretion of the small quantities of uroporphyrin (4·59 μg.) and coproporphyrin (6·85 μg.) was more protracted. A smaller yield of porphobilinogen was obtained in the urine of rat no. 2, viz. 2·85 mg., of which 2·6 mg. were excreted in the first 3 hr. after injection. This rat excreted a total of 4·95 μg. of uroporphyrin and 6·0 μg. of coproporphyrin in the 24 hr. following injection.

Paper chromatography of these porphyrins showed that they behaved as uroporphyrin III and coproporphyrin III, respectively. The porphobilinogen excreted in the urine was indistinguishable from crystalline porphobilinogen by paper chromatography, using butanol–acetic acid (Westall, 1952). Furthermore, the lactam derivatives (Cookson & Rimington, 1953) of the porphobilinogen excreted in the urine and of crystalline porphobilinogen, behaved identically on a paper chromatogram.

Table 1 summarizes the mean daily faecal coproporphyrin and protoporphyrin values before and after administration of porphobilinogen in rats nos. 1 and 2. These figures are similar in each rat. Paper chromatography of these porphyrins both before and after porphobilinogen administration demonstrated that some dicarboxylic porphyrin (similar in properties to protoporphyrin) as well as coproporphyrin had been extracted by 0·1 N-HCl and thus the coproporphyrin determinations in Table 1 are too high. However, an amount of dicarboxylic porphyrin similar to that of coproporphyrin (as judged by fluorescence on paper chromatograms) was extracted by 0·1 N-HCl in each case. It was therefore considered that no significant change had occurred in faecal porphyrins as a result of porphobilinogen administration.

Neither rat showed any abnormal symptoms during the 8-day period following the porphobilinogen injection. Haemoglobin contents, red cell numbers and reticulocyte counts were determined in both rats, before and at the end of the experiment and one day before and one day after the injection. No significant changes in these haematological values were observed.

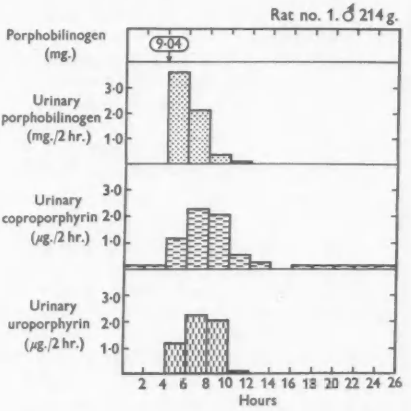

Fig. 1. Urinary excretion of porphobilinogen and porphyrins after the intravenous injection of porphobilinogen in rat no. 1.

Table 1. *Urinary and faecal porphyrin determinations made with rats nos. 1, 2 and 3, before and after porphobilinogen (Pbg.) administration, by the intravenous (i.v.) or enteral (g.i.) routes*

Urinary and faecal porphobilinogen were recovered after its administration. The durations of the 'before' periods for rats nos. 1, 2 and 3 were 8, 7 and 6 days respectively; those for 'after' were 3 days in each case.

Rat no.	Weight (g.)	Dose of Pbg. (mg.)	Mean urinary coproporphyrin (μg./day)		Mean faecal coproporphyrin (μg./day)		Mean faecal protoporphyrin (μg./day)		Pbg. recovered		
			Before	After	Before	After	Before	After	Faeces (mg.)	Urine (mg.)	% (urine + faeces)
1	266	9·04 (i.v.)	1·7 (±0·6)	3·7 (±2·2)	134 (±33)	78 (±16)	135 (±39)	130 (±31)	0·097	6·07	68
2	172	9·4 (i.v.)	1·3 (±0·4)	3·7 (±1·9)	53 (±10·7)	42 (±9·1)	98 (±25)	122 (±36)	0·055	2·85	31
3	214	3·68 (g.i.)	1·6 (±0·6)	2·1 (±1·4)	39 (±12·6)	27 (±6·7)	66 (±15·2)	79 (±29·2)	1·676	0	45

Rats killed within 30 *min.* Porphobilinogen (2·2, 2·0 and 2·14 mg.) was injected by vein into rats nos. 4–6 and these were killed after 30, 30 and 10 min., respectively. The porphobilinogen contents of the tissues of these rats are summarized in Table 2. The urine, plasma and kidney always contained porphobilinogen, while liver, spleen, brain, bone marrow and Harderian glands gave consistently negative results. Subcutaneous fat and lungs contained porphobilinogen, while skeletal and cardiac muscles (thoroughly washed free of blood) of rat no. 6 contained porphobilinogen. The plasma porphobilinogen level of rat no. 6 was approximately 4 times that of rats nos. 4 and 5 (killed at 30 min.). Small amounts of porphobilinogen were found in the gut contents.

Subcutaneous injection of porphobilinogen

Rats killed within 30 *min. after single injection.* Porphobilinogen (2·0 and 2·62 mg.) was injected into rats nos. 7 and 8, respectively, and the animals were killed 30 min. after the injection. The pattern of urinary and tissue porphobilinogen (Table 2) was similar to that found after intravenous injection. The plasma porphobilinogen level was higher than that found in rats nos. 4 and 5.

Rats killed 20·25 *hr. after half-hourly injections, maintained for* 20 *hr.* In experimental porphyria in rats induced by allyl*iso*propylacetamide (Goldberg & Rimington, 1954*b*), porphobilinogen is consistently found in the liver in high concentration, also in the kidney and urine, and sometimes in the plasma. This hepatic porphobilinogen could have been formed in the liver or at an extrahepatic site, e.g. the bone marrow, and transported by the blood stream to the liver. The very rapid excretion of porphobilinogen in the urine and the absence of porphobilinogen from the liver in the above experiments, when the plasma still contained porphobilinogen, made such an extrahepatic site unlikely. However, it was still possible that the liver might take up porphobilinogen from an extrahepatic site if porphobilinogen were present in the plasma for any length of time. To determine the approximate length of the period during which significant plasma levels are maintained in experimental porphyria, rat no. 12 was given allyl*iso*propylacetamide by gastric intubation. Within 20 hr. this rat had excreted porphobilinogen in the urine. It was killed and porphobilinogen was found in the liver and kidney (Table 2).

In order to mimic the effects of continuous production of porphobilinogen by a possible extrahepatic site throughout this period, 1 mg. of porphobilinogen was injected subcutaneously into rat no. 9 every 30 min. for 20 hr. The initial two doses were given together at the beginning of the experiment. Urinary excretion of porphobilinogen

Table 2. *Tissue porphobilinogen (Pbg.) determinations of rats* (4–11)*, given porphobilinogen, intravenously (i.v.) subcutaneously (s.c.) or by gastric intubation (g.i.)*

Rat no. 12 was given 50 mg. of allyl*iso*propylacetamide (A.I.A.). Duration refers to period between porphobilinogen or A.I.A. administration and killing of animals. Porphobilinogen results are expressed as μg. total porphobilinogen (urine and gut contents) or as μg./g. or μg./ml. (the remaining tissues); '+' indicates a trace.

Rat no.	Body wt. (g.)	Dose Pbg. (mg.)	Duration (min.)	Porphobilinogen: Urine (total)	Gut (total)	Kidney	Plasma	Liver	Skeletal muscle	Fat	Lungs	Heart muscle	Pbg. recovered (μg.)	Pbg. recovered (%)
4	150	2·2 (i.v.)	30	854	+	50	11·3	0	*	*	*	*	976	43
5	174	2·0 (i.v.)	30	805	2·5	42·5	8·3	0	0	11·3	9·1	*	966	48
6	160	2·14 (i.v.)	10	4	+	124	38	0	10·6	9·3	8	0	1 305	61
7	112	2·0 (s.c.)	30	645	+	75	27·6	0	*	+	+	8	1 186	59
8	151	2·62 (s.c.)	30	719	10	100	26·2	0	5	16·5	10·6	0	1 021	39
9	150	40·0 (s.c.)	1215	30 160	280	69·5	27·6	0	0	7·3	6·9	0	31 734	79
10	190	3·4 (g.i.)	450	168	2122	0	0	0	0	0	0	0	2 290	67
11	197	4·85 (g.i.)	120	87	1750	0	2·1	0	0	0	0	0	1 955	40
12	174	0† (g.i.)	1200	515	9·8	10·7	0	28·6	0	0	0	0	806	—
13	142	Normal control	—	0	0	0	0	0	0	0	0	0	—	—

* Not tested.

† 50 mg. A.I.A.

Table 3. *Porphyrin determinations (μg./g. or μg./ml.) in tissues of rats which had been given porphobilinogen*

Uroporphyrin was not found in any of the tissues of these rats.

Rat no.	Liver		Kidney		Blood	
	Copro-porphyrin	Proto-porphyrin	Copro-porphyrin	Proto-porphyrin	Copro-porphyrin	Proto-porphyrin
4	0·04	0·02	0·01	0·04	0	0·06
6	0·23	0·16	0·12	0·13	0·02	0·26
7	0·09	0·11	0	0·25	0	0
9	0·02	0	0·31	0·57	0	0
13 (control)	0·02	0·16	0·08	0·07	0·02	0·49

was determined hourly and urinary uroporphyrin and coproporphyrin, as well as plasma porphobilinogen were determined every 2 hr. (Fig. 2). The rat was killed 15 min. after the final injection and tissues were analysed for porphobilinogen and porphyrins (Tables 2 and 3). The liver did not contain porphobilinogen, while the gut contents showed a significant amount of porphobilinogen, presumably from passage through the bile ducts. During the 20 hr. period of the experiment the rat showed no pathological symptoms.

Inspection of Fig. 2 shows that during the latter 10 hr. of this experiment the hourly urinary excretion levels of porphobilinogen were remarkably constant—a mean of 1530 ± 151 μg. (S.D.)/hr. The mean plasma porphobilinogen level during this period was $31 \pm 7{\cdot}4$ μg. (S.D.)/ml. Thus the renal clearance for porphobilinogen in this rat was 0·82 ml./min. or 0·55 ml./min./100 g. of body weight. This figure is in good agreement with inulin clearance found in the rat (Smith, 1951) and would therefore suggest that porphobilinogen is filtered by the glomeruli and not reabsorbed to a significant extent.

Table 3 summarizes the liver, kidney, and blood porphyrin determinations of rats nos. 4, 6, 7 and 9, which had been given porphobilinogen parenterally. There is no significant elevation of the porphyrin levels.

Enteral administration of porphobilinogen

Excretion pattern of porphobilinogen and porphyrins. A study similar to that described for rats nos. 1 and 2, was carried out in rat no. 3. After a preliminary period of 6 days, 3·68 mg. of porphobilinogen were given by gastric intubation. Daily urinary and faecal porphobilinogen and porphyrin contents were determined during the base-line period and in the 7 days following porphobilinogen administration (Table 1). There was no significant change in the porphyrin levels in the urine or faeces. No porphobilinogen appeared in the urine of this rat (cf. rats nos. 10 and 11, below), but 45% of the porphobilinogen administered was

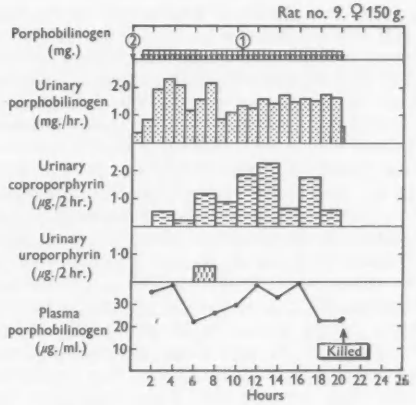

Fig. 2. Effects of repeated subcutaneous injections of porphobilinogen for 20 hr. on urinary porphobilinogen and porphyrin excretion and on plasma porphobilinogen. For experimental details see text.

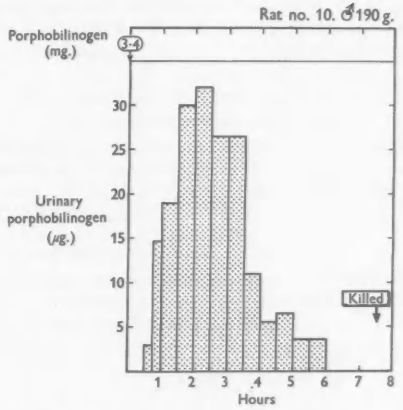

Fig. 3. Urinary excretion of porphobilinogen after intragastric intubation of 3·4 mg. of porphobilinogen.

recovered in the faeces. Traces of porphobilinogen were present in the faeces up to the sixth day after porphobilinogen administration.

Rat killed after 7·5 hr. Rat no. 10 was given 3·4 mg. of porphobilinogen by gastric intubation. Urinary porphobilinogen determinations were done every 30 min. (Fig. 3). Porphobilinogen was detected in the urine within the second half-hour and was excreted in small amounts until 6 hr. after its administration. Maximum excretion in the urine occurred between 2 and 2·5 hr. Between 6 and 7·5 hr., when the rat was killed, no porphobilinogen was detected in the urine. After death 2·12 mg. of porphobilinogen was recovered from the intestinal contents. It was notable that in spite of this large quantity of porphobilinogen in the gut, it was not excreted in the urine in the last 1·5 hr. and this would suggest that porphobilinogen is only absorbed in the upper part of the rat intestine. Porphobilinogen was not found in the other tissues (Table 2).

Rat killed after 2 hr. Rat no. 11 was given 4·85 mg. of porphobilinogen by gastric intubation and killed 2 hr. later in order to see whether porphobilinogen could be detected in the liver during a phase of maximal absorption of porphobilinogen from the gut (see above). Urinary porphobilinogen determinations, as in rat no. 10, showed the excretion of 11·3, 30 and 46 μg. of porphobilinogen in the second, third and fourth half-hour periods, respectively, after administration. The rat was then killed and 1·75 mg. of porphobilinogen were recovered from the intestinal contents. The plasma contained 2·1 μg. of porphobilinogen/ml., whilst 0·104 mg. of porphobilinogen was recovered from gut tissues after they had been thoroughly washed with normal saline. Porphobilinogen was not present in liver, kidney, muscle, fat, Harderian gland or brain (Table 2). No porphyrins were detectable in the liver.

In vitro *incubation of porphobilinogen with gastro-intestinal contents of a rat*

This experiment was carried out to investigate further the absence of conversion of porphobilinogen into porphyrins in the gut, which was demonstrated in rat no. 3. A rat was killed and its gastro-intestinal contents were mixed with sterile normal saline to give a total vol. of 23 ml. 4·5 ml. of this were pipetted into each of four 50 ml. conical flasks. Flasks nos. 1 and 2 were immersed in boiling water for 5 min. and 1 mg. of porphobilinogen in 0·56 ml. of sterile normal saline was added to flasks nos. 2 and 4. The pH of each of the contents of these flasks was about 6·6. The flasks were then plugged with cotton wool and incubated for 4 hr. with shaking at 90–100 oscillations/min. at 37°. The contents were then analysed for porphobilinogen and porphyrins (Table 4).

The results show that a 59 and 52% recovery of porphobilinogen was achieved from flasks nos. 2 and 4, but there was no significant change in the porphyrin content in any flask, with the exception of a small quantity of uroporphyrin (0·71 μg.) found in flask no. 2.

DISCUSSION

The results show that most of the porphobilinogen administered parenterally is excreted in the urine and that this excretion is rapid; small amounts, however, are recovered in the faeces. Given, however, by the enteral route, it is mainly excreted unchanged in the faeces, although small amounts, (in the case of rat no. 10, one-twentieth of the porphobilinogen given) may be excreted in the urine. The excretion behaviour of the naturally occurring porphyrins (uroporphyrin, coproporphyrin and protoporphyrin), after enteral and parenteral administration to experimental animals, has been studied previously. Fischer (1916) and Günther (1922) found that uroporphyrin given parenterally to mice and rabbits, respectively, is mainly excreted in the urine, while Fischer (1915) found that uroporphyrin, when taken by himself by mouth, was passed unchanged in the stool. On the other hand, coproporphyrin III, given orally or parenterally to rats, is not passed in the urine, but recovered in the stool (Hoffbauer, Watson & Schwartz, 1953). Protoporphyrin injected parentally into dogs with a bile-renal fistula was not accounted for in the porphyrins excreted thereafter, apart from traces of material resembling deuteroporphyrin (Watson, Pass & Schwartz, 1941). The excretion of porphobilinogen thus resembles more that of uroporphyrin than that of coproporphyrin or protoporphyrin. The mean percentage recovery of the porphobilinogen administered, parenterally or enterally, was 53 ± 14. This compares with the 50% recovery of

Table 4. *Incubation of porphobilinogen with gastro-intestinal contents of a rat*

An equal volume of a mixture of gastro-intestinal contents in sterile 0·9% (w/v) NaCl was added to each of flasks nos. 1–4. Flasks nos. 1 and 2 were immersed in boiling water for 5 min. before incubation.

Flask no.	Porphobilinogen added before incubation (mg.)	Porphobilinogen recovered after incubation (mg.)	Uroporphyrin (μg.)	Coproporphyrin (μg.)	Protoporphyrin (μg.)
1	0	0	0	0·16	4·10
2	1	0·59	0·71	0·36	2·90
3	0	0	0	0·29	6·25
4	1	0·52	0	0·26	2·75

coproporphyrin III injected parenterally into rats by Hoffbauer *et al.* (1953).

The absence of abnormal symptoms in any of the rats used in the present work confirms the results of the pharmacological study of porphobilinogen by Goldberg *et al.* (1954). It is of some interest that a small, though significant, rise of coproporphyrin III occurred in the urine of rats nos. 1, 2 and 9, besides some excretion of uroporphyrin III. This is in accordance with the demonstration by Falk *et al.* (1953) using a haemolysed chicken erythrocyte system, that porphobilinogen is a precursor of uroporphyrin, coproporphyrin and protoporphyrin.

The site of the excessive porphobilinogen formation in acute porphyria is of importance. Prunty (1945) showed that porphobilinogen is found in high concentration in the liver at autopsy of these cases, and this observation has been repeatedly confirmed. Thus Schmid, Schwartz & Watson (1954) include this disease in their 'porphyria hepatica' group. Porphobilinogen is also found in the kidney and urine and sometimes in the bile and plasma, but not in other tissues. In experimental animals drugged with sedormid or allyl*iso*propylacetamide, these same tissues also contain porphobilinogen and in the same relative concentrations. In fact Schmid & Schwartz (1952) refer to the experimental porphyria produced by sedormid as belonging to the 'hepatic type'. This suggested but did not prove, that in acute porphyria and in experimental porphyria of animals, porphobilinogen is formed in the liver. It did not exclude the possibility that porphobilinogen is formed at an extrahepatic site, e.g. the bone marrow, and is transported by the blood stream to the liver. The present experiments indicate that porphobilinogen is mainly excreted by glomerular filtration in the rat. Further, they show that porphobilinogen is not found in the liver after enteral or parenteral administration. The plasma porphobilinogen levels in these experiments were high, higher than those found in an attack of acute porphyria. In rat no. 9 this plasma level was maintained for 20 hr., a period which had been found sufficient to induce an experimental porphyria by means of allyl*iso*propylacetamide, with accumulation of porphobilinogen in the liver in rat no. 12. These facts would suggest that extrahepatic porphobilinogen formation in experimental porphyria in the rat is improbable. It is also of interest that there was no significant increase of either coproporphyrin or protoporphyrin in the livers of rats given porphobilinogen. In the livers of patients with acute porphyria and of animals with experimental porphyria such an increase is present.

There is some evidence that the mechanism of porphobilinogen excretion in the rat may be relevant to human acute porphyria. In this disease, the high porphobilinogen concentrations in the urine and the persistently low concentrations in the plasma, even at the height of an attack, suggest a rapid excretion of that substance. Furthermore, a sharp rise of porphobilinogen excretion in the urine often accompanies and may even precede the onset of clinical symptoms of porphyria, which would also suggest rapid elimination. Finally, acute porphyria urine, which is freshly passed and has not lain in the bladder for long, contains mainly porphobilinogen with only traces of coproporphyrin and uroporphyrin. This is the same pattern of porphobilinogen and porphyrin excretion as is found in the urine of a rat, injected parenterally with porphobilinogen. A greater conversion of porphobilinogen into porphyrins would be expected, if porphobilinogen were not rapidly eliminated in acute porphyria. The probability of such a rapid elimination would explain the absence of photosensitivity in this disease, since this symptom is caused by an excessive amount of formed porphyrins in skin. These findings would suggest that the renal excretion of porphobilinogen in the rat is similar to that in human acute porphyria.*

It has been pointed out that the porphobilinogen content of tissues in human acute porphyria and in experimental porphyria in animals is similar. This might suggest, in addition, that the reasoning put forward for a hepatic site of formation of porphobilinogen in the rat is valid for the human disease.

SUMMARY

1. Porphobilinogen has been administered parenterally and enterally to rats. After parenteral injection, porphobilinogen was rapidly and mainly excreted in the urine. Small amounts were excreted in the faeces. After enteral administration, only small amounts were slowly excreted in the urine. Most of it was passed unchanged in the faeces. The porphobilinogen excreted in urine was identical chromatographically with that administered.

2. A calculation of renal clearance of porphobilinogen in the rat suggests that porphobilinogen is mainly excreted by glomerular filtration without significant reabsorption.

3. A small but significant rise of coproporphyrin III, as well as the excretion of some uroporphyrin III, was noted in rat urine after parenteral injection of porphobilinogen. There was no significant change in faecal porphyrin excretion after enteral or parenteral administration of porphobilinogen.

4. Aerobic incubation *in vitro* at 37° of porphobilinogen with gastro-intestinal contents of a rat

* *Note added in proof.* Renal clearance studies recently done by the author in human acute and latent porphyria have shown that the endogenous porphobilinogen is excreted in man by glomerular filtration without significant reabsorption.

showed no evidence of conversion of porphobilinogen into porphyrins.

5. Porphobilinogen was not found in the liver when rats were killed shortly after its enteral or parenteral administration, nor was it found in the liver of a rat after subcutaneous injections of porphobilinogen every half-hour for 20 hr., during which time a high plasma porphobilinogen concentration was maintained. These findings suggest that the porphobilinogen found in the liver of a rat with experimental porphyria is in fact formed there and is not transported by the blood stream to the liver from an extrahepatic site.

6. The relevance of these results, obtained in the rat, to human acute porphyria, is discussed.

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The Free Energy Change Associated with the Hydrolysis of the Thiol Ester Bond of Acetyl Coenzyme A

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In previous papers (Burton & Wilson, 1953; Burton & Krebs, 1953), free-energy data were evaluated for the individual reactions of glycolysis, alcoholic fermentation and the tricarboxylic acid cycle. Two approximate values were given for the free energy of hydrolysis of the thiol ester bond of acetyl coenzyme A (acetyl CoA), viz. $-13{\cdot}4$ and $-11{\cdot}5$ kcal. at pH 7·5.

These estimates were revisions of earlier ones obtained by Stadtman (1952) and by Stern, Ochoa & Lynen (1952) and were based respectively on their equilibrium data for the phosphotransacetylase and the condensing enzyme reactions. Some of the free-energy data used in all these calculations are now known to be seriously in error. Revised calculations described in this article lead to $\Delta G'$ values of approximately -8 kcal., and these are shown to be consistent with free-energy data for the acetaldehyde-oxidizing system of *Clostridium kluyveri* (Burton, R. M. & Stadtman, 1953).

Revised values are also given for the free energies of formation of the tricarboxylic acids, and the reliability of the previously reported data for the terminal pyrophosphate group of adenosine triphosphate (ATP) is briefly discussed.

Thermodynamic symbols and conventions

These are the same as used previously (Burton & Krebs, 1953). ΔG° is the free energy change for conditions where all the reactants including the H^+ ion are in their thermodynamic standard states. $\Delta G'$ is for conditions where all the reactants except

the H^+ ion are in their standard states. If the H^+ or OH^- ions are not reactants, $\Delta G'$ is the same as $\Delta G°$. The standard state for water is the pure liquid and for carbon dioxide is the gas at 1 atm.

Free energy of hydrolysis of acetyl CoA at the thiol ester bond

Previous calculations based on the equilibrium of the condensing enzyme system (Stern *et al.* 1952; Burton & Krebs, 1953), are now known to contain an error of 5·46 kcal. This is due to an incorrect value for the free energy of formation ($\Delta G°f$) of citrate resulting from an arithmetical error in the equilibrium constant of the *iso*citrate dehydrogenase reaction. This error appeared in Ochoa's (1948) calculation from his experimental results and was pointed out by Harary, Korey & Ochoa (1953).

The revised calculation for the condensing enzyme system using the equilibrium data of Stern *et al.* is shown in the accompanying table. The value of $\Delta G'$ thus obtained for the hydrolysis of acetyl CoA is −7·38 kcal. at pH 7.

Because of this arithmetical error, the previously reported $\Delta G°f$ values for citrate, *cis*-aconitate and *iso*citrate have to be revised and made more negative by 5·46 kcal. The revised $\Delta G°f$ values are −279·36, −220·63 and −277·77 kcal., respectively.

It is not certain what value to accept for the $\Delta G°f$ of oxalosuccinate. The value previously reported was obtained from the value for *iso*citrate and from $K = [\text{D-}iso\text{citrate}]\ [\text{TPN}_{ox.}]/[\text{oxalosuccinate}]\ [\text{TPN}_{red.}] = 0{\cdot}3$ at pH 7·2 and 23° (Ochoa, 1948). To be consistent, the $\Delta G°f$ value for oxalosuccinate should also be revised by 5·46 kcal. to give −274·2 kcal. However, the corresponding free energy changes for the decarboxylation of oxalosuccinate to α-oxoglutarate and also for the reduction to *iso*citrate are appreciably different from the values for the analogous reactions of oxaloacetate.

From Stadtman's (1952) data for the phosphotransacetylase equilibrium and from Korey's unpublished equilibrium data for the acetokinase reaction, Korkes (1952) concluded that $\Delta G°$ is practically zero for the reaction:

$$ATP^{4-} + \text{acetate}^- + \text{CoA} \rightarrow ADP^{3-} + \text{acetyl CoA} + HPO_4^{2-}.$$

This means that $\Delta G'$ for the hydrolysis of acetyl CoA is practically the same as that for the hydrolysis of ATP to adenosine diphosphate (ADP) and inorganic phosphate. After considering the data available for the calculation of the free energy of hydrolysis of ATP, Burton & Krebs (1953) obtained the value of $\Delta G' = -9{\cdot}4$ kcal. at pH 7·5. This would therefore indicate that $\Delta G'$ for the hydrolysis of acetyl CoA is −9·4 kcal. at pH 7·5 or −8·7 kcal. at pH 7·0. The similar calculations which have been previously reported (Korkes, 1952; Burton & Krebs, 1953) have given numerically larger values either because of using a different value for the hydrolysis of ATP or because an incorrect value was assumed for the acetokinase equilibrium.

From their equilibrium data for the CoA-dependent acetaldehyde-oxidizing system of *Cl. kluyveri*, Burton (R. M.) & Stadtman (1953) obtain two different values for the hydrolysis of acetyl CoA, depending on the ancillary free-energy data used. In this calculation the ancillary data required are the $\Delta G°f$ values for aqueous acetaldehyde and the acetate ion together with the free energy of reduction of diphosphopyridine nucleotide (DPN). The data for acetaldehyde and DPN as given by Burton & Wilson (1953) lead to the value of $\Delta G' = -8{\cdot}25$ kcal., for the hydrolysis of acetyl CoA at pH 7·0. The other set of ancillary data used by Burton (R. M.) & Stadtman gave a value of

Table 1. *Calculation of the free energy of hydrolysis of acetyl* CoA

$$\text{Acetyl CoA} + H_2O \rightarrow \text{CoA} + \text{Acetate} + H^+.$$

Reaction *A*	$\Delta G'$ (kcal.)	Reaction *B*	$\Delta G'$ (calc.) (kcal.)	$\Delta G'$ of hydrolysis of acetyl CoA (kcal.)	
				(*B* − *A*)	(pH 7·0)
$\text{Citrate}^{3-} + \text{CoA} + H^+ \rightleftharpoons \text{oxaloacetate}^{2-} + \text{acetyl CoA}$	7·72 (pH 7·2)	$\text{Citrate}^{3-} \rightleftharpoons \text{oxaloacetate}^{2-} + \text{acetate}^-$	0·07*	−7·65 (pH 7·2)	−7·38
$ATP + \text{acetate}^- + \text{CoA} \rightleftharpoons ADP + \text{acetyl CoA} + HPO_4^{2-}$	0	$ATP^{4-} + H_2O + H^+ \rightleftharpoons ADP^{3-} + HPO_4^{2-}$	−9·4† (pH 7·5)	−9·4 (pH 7·5)	−8·7
$\text{Acetaldehyde} + \text{CoA} + DPN^+ \rightleftharpoons \text{acetyl CoA} + DPNH + H^+$	−4·22 (pH 7·0)	$\text{Acetaldehyde} + DPN^+ \rightleftharpoons DPNH + \text{acetate}^- + H^+$	−12·47‡ (pH 7·0)	−8·25 (pH 7·0)	−8·25

* Calculated from the $\Delta G°f$ values for oxaloacetate and acetate (Burton & Krebs, 1953) and the $\Delta G°f$ value for citrate given in the text.

† Burton & Krebs (1953).

‡ Calculated from data for acetaldehyde and the reduction of DPN (Burton & Wilson, 1953) and the $\Delta G°f$ value for acetate (Burton & Krebs, 1953).

$-13{\cdot}04$ kcal. This value is inaccurate, since the $\Delta G°f$ value for acetaldehyde (Parks & Huffman, 1932) is based on an incorrect heat of combustion of ethanol, and also the value (Borsook, 1940) taken for the free energy of reduction of DPN is incorrect.

Of the three values, that of $-8{\cdot}25$ kcal. obtained from the data for the acetaldehyde oxidizing system of *Cl. kluyveri* appears to be the most reliable; the most serious uncertainty is that introduced by the data on the heat of combustion of acetic acid. This particular uncertainty is also introduced into the value obtained from the condensing enzyme system ($-7{\cdot}4$ kcal.), but it does not apply to the value obtained from the phosphotransacetylase equilibrium and the free energy of hydrolysis of ATP ($-8{\cdot}7$ kcal.). Both of these latter calculations are somewhat lengthy, and it is possible that small cumulative errors are introduced at several of the steps.

DISCUSSION

The consistency of the three values obtained for the $\Delta G'$ of hydrolysis of the thiol ester bond of acetyl CoA supports the reliability of the free-energy data on which they are based. Two recent independent measurements have confirmed some of the free-energy data which were previously reported. Rodkey (1954) has measured the oxidation–reduction potential of DPN and obtained $E'_0 = -0{\cdot}318$ v at pH 7 and 30°. Equilibrium measurements by Harary *et al.* (1953) for the 'malic enzyme' system give a $\Delta G°$ value of 0·20 kcal., which is very close to that of 0·18 kcal. which was evaluated indirectly by Burton & Krebs. This agreement confirms the $\Delta G°f$ value obtained for pyruvate. It should be noted that the standard state for carbon dioxide in these $\Delta G°$ values is gaseous carbon dioxide at 1 atm., whereas Harary *et al.* used 1 M solution as the standard state.

In the data for the steps of glycolysis, there remains an uncertainty in the equilibrium data for the pyruvate–phosphokinase reaction. Utter & Kurahashi (1954) have described the reaction

$$\text{enol pyruvate 2-phosphate}^{3-} + \text{IDP}^{3-} + \text{H}^+ + \text{HCO}_3^- \rightleftharpoons \text{oxaloacetate}^{2-} + \text{ITP}^{4-}.$$

They report $\Delta G°$ to be $-9{\cdot}27$ kcal. If, as is likely, the free energy of hydrolysis of the terminal pyrophosphate group of inosine triphosphate (ITP) is the same as that for ATP, this $\Delta G°$ value, together with the previously reported $\Delta G°f$ values for oxaloacetate, pyruvate and bicarbonate indicate that $\Delta G°$ for the pyruvate–phosphokinase reaction is $-15{\cdot}66$ kcal., instead of $-14{\cdot}8$ kcal. as previously used. This would give a value of $-8{\cdot}9$ kcal. (instead of $-9{\cdot}4$ kcal.) for the $\Delta G'$ of hydrolysis of ATP to ADP and inorganic phosphate at pH 7·5. At present the 8·9 kcal. value seems to be preferable and this would, with the phosphotransacetylase data be consistent with a $\Delta G'$ of $-8{\cdot}2$ kcal. (pH 7) for the hydrolysis of acetyl CoA.

SUMMARY

1. Three partially independent calculations are reported for the free energy of hydrolysis of acetyl coenzyme A using free-energy data previously reported by Burton & Krebs (1953). The three calculations are based on the equilibria of (*a*) the condensing enzyme system, (*b*) phosphotransacetylase, and (*c*) the acetaldehyde-oxidizing system of *Clostridium kluyveri*. The values obtained are, at pH 7, $\Delta G' = -7{\cdot}38$, $-8{\cdot}7$ and $-8{\cdot}25$ kcal. respectively and are considered to be reasonably consistent. At present, a value of about $-8{\cdot}2$ kcal. appears to be the most reliable. This is consistent with the phosphotransacetylase data and a slightly revised value of $\Delta G' = -8{\cdot}9$ kcal. for the hydrolysis of the terminal pyrophosphate group of adenosine triphosphate at pH 7·5.

2. Revised values for the free energies of formation ($\Delta G°f$) of the citrate^{3-}, *cis*-aconitate^{3-} and D-*iso*citrate^{3-} ions are respectively $-279{\cdot}36$, $-220{\cdot}63$ and $-277{\cdot}77$ kcal.

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The Distribution of Diphtheria Antitoxin in Pepsin-digested Horse Antiserum

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It has been shown by many workers, both by salting-out methods and by electrophoresis, that antibodies to a single antigen are not confined to any particular fraction of the serum proteins, and that the immunological properties of the antibody may vary from fraction to fraction. Barr, Glenny & Pope (1931) showed that addition of euglobulin to diphtheria antitoxic serum, before salting out with ammonium sulphate, caused antitoxin to be precipitated by a lower concentration of ammonium sulphate than is necessary for antiserum alone; in the presence of added albumin more ammonium sulphate than normal is required to precipitate antitoxin, that is, there is an apparent redistribution of antitoxin when the proportions of the serum proteins are altered. Barr & Glenny (1931) showed that serum protein fractions obtained by successive precipitation with increasing amounts of ammonium sulphate carried antitoxin fractions which had decreasing *in vivo/in vitro* ratios. Van der Scheer & Wyckoff (1940*a*, *b*) and Van der Scheer, Wyckoff & Clarke (1940, 1941*b*) reported that, during the immunization of horses with bacterial toxins, a new serum component, *T*, with an electrophoretic mobility between those of γ- and β-globulins, made its appearance and that part of the antitoxin was associated with it. Immunization with polysaccharide or vaccine antigens did not evoke production of a *T* component but increased the γ-globulin fraction.

By electrophoretic analysis Kekwick & Record (1941) found that during immunization of horses with diphtheria toxin the γ-globulin fraction increased slightly, but in the early stages only, and that the β-globulin rose slowly at first and then more rapidly and to a greater extent in the later stages of immunization. Antitoxic activity was associated with both fractions but their immunological properties were different. The β-antitoxin had a long flocculation time (147 min. at the 7·5 Lf/ml. level), a low *in vivo/in vitro* ratio (0·9) and gave flocules containing only half the amount of antibody nitrogen found in floccules from the γ-antitoxic fraction, which had a short flocculation time (7 min. at the 7·5 Lf/ml. level) and a high *in vivo/in vitro* ratio (2·0). The more soluble fraction (pseudoglobulin) obtained by salt precipitation contained a higher proportion of β-globulin than the less-soluble fraction (euglobulin) which contained more γ-globulin. The β-globulin of some normal horses contains two components, β_1 and β_2, whilst all the immunized horses examined had both β_1 and β_2; the increase in globulin content was associated with the slower, β_2-globulin, which corresponds to the *T* component of Van der Scheer *et al.* Kekwick, Knight, Macfarlane & Record (1941) showed that the peptic digestion of antitoxic sera by Pope's (1939) method, with a yield of about 80% of the antitoxin, gave a product having the short flocculation time of γ-globulin, but the low *in vivo/in vitro* ratio of β-globulin, which they regarded as the source of the digested antitoxin. They found that rapidly flocculating raw sera with a high γ-globulin content gave poor yields of antitoxin after digestion, and that, from a serum rich in γ-globulin, only 15–20% of the antitoxin could be recovered. They suggested that peptic digestion eliminated most of the antitoxin associated with the γ-globulin and that the product was mainly a modified β-globulin. This opinion was based on the low *in vivo/in vitro* ratios and the low yields from sera from horses in the early stages of immunization in which most of the activity was associated with γ-globulin; the digestion products were not examined electrophoretically.

By electrophoresis Van der Scheer & Wyckoff (1940*c*) followed the digestion of horse antidiphtheria and antitetanus sera with pepsin at pH 4 and 37°, and found that the *T* peak, characteristic of antitoxin, disappeared within 10–30 min. and was replaced by one with a mobility corresponding to that of γ-globulin. The albumin was also rapidly destroyed. This was confirmed by Van der Scheer, Wyckoff & Clarke (1941*c*), who showed in addition that prolonged digestion reduced the amount of material constituting the newly formed γ peak. There was a 69% loss of coagulable protein and a 20% loss of antitoxic activity. When antiserum was heated at 37° at pH 4 in the absence of pepsin the γ-globulin peak, originally present, disappeared and was replaced by *T* or $T+T_1$ (T_1 having a mobility between those of γ and *T*). The globulins constituting this fraction were readily digested by pepsin. The T_1 component was regarded as being

due to aggregation of the γ-globulin; it could be precipitated on neutralization to pH 7 but redissolved at pH 7·6. Heating normal or antitoxic plasma at 53–65° caused the appearance of a new peak, *C*, with a mobility about the same as that of β-globulin (Van der Scheer, Wyckoff & Clarke, 1941*a*). The *C* peak of antitoxic plasma was formed at the expense of the γ and *T* components and was only slowly digested by pepsin.

Fell, Stern & Coghill (1940) reported that antitoxic horse sera had increased β- or γ-globulin fractions and that pepsin-digested antitoxins consisted mainly of β- and γ-globulins. Modern & Ruff (1938*a*, *b*, 1940*a–d*, 1942) showed that almost all the albumin and 80 % of the euglobulin of normal and antitoxic horse sera, together with 75 % of the pseudoglobulin of normal sera and 66 % of that of antitoxic sera, were destroyed by peptic digestion at pH 3·8 and 45°. They considered it probable that most of the antitoxic activity lost on digestion was that associated with the less-resistant euglobulin. Harms (1948) digested euglobulin and pseudoglobulin fractions obtained by ammonium sulphate precipitation of horse diphtheria antitoxin with pepsin at pH 3·2 and found that a large proportion of the euglobulin fraction was precipitated by the subsequent heating in presence of ammonium sulphate, and considerably less from the treated pseudoglobulin fraction. He considered that this precipitated globulin was mainly γ-globulin and that the remaining antitoxin was largely β-globulin, although not all the β-globulin was antitoxin. The initial fractions were examined electrophoretically and shown to be very inhomogeneous, but the composition of the final digestion products was not investigated apart from nitrogen estimations. By electrophoresis, Amoureux & Yeu (1951) showed that diphtheria and tetanus antitoxic sera from hyperimmunized horses had high β-globulin and low γ-globulin contents, but that the enzyme-purified products consisted of almost pure γ-globulin. Wilson & Miles (1946) state that the enzyme digestion methods of purification of antitoxic sera favour 'the retention of the β- at the expense of the γ-antitoxin'.

Because of these discrepant opinions and because enzyme-purified antitoxins routinely produced at this Institute consist mainly of a component with the mobility of γ-globulin, it was thought desirable to follow the changes in the electrophoretic components of diphtheria-antitoxic horse serum during the various stages of preparation.

METHODS

A pool of plasma from eleven horses hyperimmunized with diphtheria toxin for the routine production of diphtheria antitoxin was used throughout. It had 850 antitoxin units/ml., 15·34 mg. total N/ml., 0·31 mg. non-protein N/ml., 15·03 mg. protein N/ml. and 56·5 units/mg. protein N.

Fractionation of plasma

Ammonium sulphate. For comparative purposes the method of fractionation described by Harms (1948) was adopted. Plasma was diluted with an equal volume of water and a euglobulin fraction obtained by the addition of 22 g. of $(NH_4)_2SO_4$/100 ml. The precipitated globulin was separated by centrifuging and a pseudoglobulin fraction was precipitated by increasing the concentration of $(NH_4)_2SO_4$ in the supernatant fluid to 36 g./100 ml. After separation of this material, albumin was precipitated by bringing the $(NH_4)_2SO_4$ concentration to 55 g./100 ml. All the precipitates were dialysed against 0·9 % (w/v) NaCl solution until free from $(NH_4)_2SO_4$.

Dialysis. To a sample of plasma were added 1·2 ml. of 56 % (w/v) $CaCl_2$ solution/100 ml. and the clot was removed by centrifuging. The serum was dialysed against three changes of 15 vol. of tap water and finally against two changes of 30 vol. of distilled water. The precipitated euglobulin was separated by centrifuging and taken up in 0·9 % (w/v) NaCl solution. The supernatant fluid, containing pseudoglobulin and albumin, was not further fractionated.

Digestion. The raw plasma was treated by the routine method used to produce enzyme-purified antitoxins at these laboratories. The plasma, containing 0·3 % (w/v) of tricresol, was diluted with 2 vol. of tap water and the pH brought to 3·25. 1 in 10 000 pepsin (Armour & Co., Chicago) (2·5 g./l. of plasma) was added, the mixture was allowed to stand for 2 hr. at room temperature (about 20°), then 14 g. $(NH_4)_2SO_4$ were added/100 ml., and the pH was adjusted to 4·30. After heating the solution for 2 hr. at 55°, a further 3·5 g. of $(NH_4)_2SO_4$/100 ml. were added and the precipitate formed during the heat treatment was removed by filtration. $(NH_4)_2SO_4$ was added to the filtrate to bring the concentration to 31 % (w/v). The precipitated antitoxic fraction was filtered off and dialysed against running tap water at about 4° for 8 days.

The plasma fractions were treated in essentially the same way, quantities and times being modified to agree with those described by Harms (1948) in order to make direct comparison with his results possible. The solution containing the fraction was diluted with water containing an appropriate amount of tricresol to give a final concentration of 2·5 % of protein and 0·12 % of tricresol, and the pH value adjusted to 3·25 with HCl. Armour's 1 in 10 000 pepsin (0·5 g./100 ml.) was added and the mixture kept at room temperature for 1 hr. $(NH_4)_2SO_4$ (13 g./100 ml.) was then added, and then NaOH solution to pH 4·25. The inactive material precipitated by heating the solution to 55° for 1 hr. was removed by centrifuging and $(NH_4)_2SO_4$ added to the supernatant fluid to give a concentration of 31 % (w/v). After standing at room temperature for 1·5 hr., the precipitated antitoxic protein was separated by centrifuging, taken up in 0·9 % (w/v) NaCl solution and dialysed until free from $(NH_4)_2SO_4$. The supernatant fluid was also dialysed, first against tap water and finally against 0·9 % (w/v) NaCl solution, until free from $(NH_4)_2SO_4$.

Samples were taken immediately after the peptic digestion, after the first $(NH_4)_2SO_4$ and heat treatment, after the second $(NH_4)_2SO_4$ precipitation and from the final supernatant fluid. Where necessary the samples were brought to pH 7 and dialysed to remove $(NH_4)_2SO_4$.

Table 1. *The results of fractionation of diphtheria antitoxic plasma*

The values are weights of the components/100 ml. of original plasma.

	Components (g./100 ml. plasma)						Antitoxin	
		Globulins						
Fraction	Albumin	α-	β-	*T*-	γ-	Total	Units/ml.	% recovery
Original plasma	1·45	1·17	1·48	3·25	2·05	9·40	850	100
Salt precipitated								
Euglobulin	0·23	0·43	0·83	2·66	1·53	5·68	550	77·7
Pseudoglobulin	0·89	0·45	0·33	0·52	0·27	2·46	250	21·2
Albumin	0·30	0·04	0	0	0	0·34	2	0·1
Total	1·42	0·92	1·16	3·18	1·80	8·48	—	99·0
Dialysis								
Water-insol. euglobulin	0	0	0·18	0·52	1·10	1·80	115	3·4
Water-sol. pseudoglobulin + albumin	1·01	0·64	0·87	2·14	1·25	5·91	660	78·9
Total	1·01	0·64	1·05	2·66	2·35	7·71	—	82·3

Total and non-protein N values and antitoxic potency were determined for the original plasma and fractions and for the samples taken at various stages of digestion by the usual methods.

Electrophoresis. The solutions for electrophoresis were diluted in phosphate buffer solution of pH 7·5 and ionic strength 0·1 to contain 1·5% (w/v) of protein and dialysed against a large volume of the same buffer solution overnight in the refrigerator. Electrophoresis was carried out in the Tiselius apparatus at 130 v and 10 ma at 1°. For evaluation, the photographed curves were enlarged by projection and traced on paper. The areas under each peak were measured with a planimeter and the proportion of each component was calculated.

RESULTS

The results of fractionation are collected in Table 1, from which it can be seen that antitoxin is associated with both euglobulin and pseudoglobulin salt-precipitated fractions in the proportions 77·7 and 21·2% respectively. The euglobulin and pseudoglobulin obtained by dialysis not only have proportions of components very different from each other but also from the salt-precipitated fractions and a quite different distribution of the antitoxic activity, 3·4% being in the water-insoluble euglobulin and 78·9% in the water-soluble pseudoglobulin. It is interesting to note that the proportions of β-, *T*- and γ-globulins in salt-precipitated euglobulin are very close to those in the water-soluble pseudoglobulin fraction. The final products after digestion, heat treatment and ammonium sulphate precipitation all have approximately the same proportional composition whether derived from raw plasma or from any of the fractions, and contain about 70% of γ-globulin and 20% of β- and *T*-globulins (Table 2). The recovery of β- + *T*-globulins and of γ-globulin after digestion of raw plasma and of different fractions is shown in Table 3. It is obvious that the recovery of γ-globulin is always greater, and usually very much greater, than that of β- + *T*-globulins.

The recovery of antitoxic activity (see Table 2) from raw plasma and from the salt-precipitated fractions is about 50–60%, but there is a wider difference in the recoveries from water-insoluble euglobulin (45·4%) and water-soluble pseudoglobulin (70·6%). The increase in purity, that is the ratio of antitoxin units/mg. of protein nitrogen before and after digestion, is fairly constant at 2·5 to 3 times.

DISCUSSION

During the heat denaturation stage of the process, the proportion of material having the mobility of γ-globulin increases and that of the other components diminishes; the loss is particularly associated with the β and *T* components. This becomes very obvious when the results are expressed as percentage recoveries of the amounts originally present in the starting material, as shown in Table 3. This finding is similar to those of Van der Scheer & Wyckoff (1940*c*), of Van der Scheer *et al.* (1941*a*) and of Amoureux & Yeu (1951) on digested plasma and heat-treated plasma, but is in conflict with the statements of Harms (1948) and of Wilson & Miles (1946).

After the fractionation of plasma by dialysis, the total absolute amount of γ-globulin present was greater than that in the original plasma, whilst the amount of the other globulin components was less (Table 1). Similar absolute increases in the amount of γ-globulin occurred during the digestion of raw plasma and of the water-soluble pseudoglobulin + albumin fraction and during the heat-denaturation stage of treatment of salt precipitated euglobulin (Table 2). Part of this increase during digestion is due to a component of the added pepsin, of which approximately two-thirds has the same mobility as

Table 2. *Results of peptic digestion of plasma and fractions*

Fraction	Components (g./100 ml.)						Protein nitrogen		Antitoxic activity		
		Globulins									
	Albumin	α-	β-	T-	γ-	Total	mg./ml.	% of original	Units/ml.	% recovery	Units/mg. protein N
Plasma	1·45	1·17	1·48	3·25	2·05	9·04	15·03	100	850	100	56·5
Immediately after digestion	0·57	1·32	1·26	1·80	3·21	8·16	4·35	28·9	230	81·5	53·0
After heat denaturation	0	0·15	0·36		1·05	1·56	0·83	5·5	190	67·5	230
Final product	0	0·13	0·37		1·24	1·74	33·40	—	5700	55·6	170
Discarded supernatant	—	—	—	—	—	—	—	—	1·5	—	—
Salt-precipitated euglobulin	0·19	0·36	0·73	2·22	1·28	4·78	7·63	100	550	100	72
Immediately after digestion	0	2·50		2·32		4·82	3·85	50·4	214	77·8	55·5
After heat denaturation	0	0	0	0·72	2·88	3·60	2·87	37·5	166	60·3	58
Final product	0	0	0	0·23	0·80	1·03	3·58	—	500	41·8	140
Discarded supernatant	0	0	0	0·02	0·04	0·06	0·04	—	0·2	0·1	5
Salt-precipitated pseudoglobulin	1·24	0·62	0·46	0·73	0·37	3·42	5·46	100	250	100	73
Immediately after digestion	0·56	0·38	0·19	0·20	0·65	1·98	3·57	65·3	155	82·8	43·4
After heat denaturation	0	0·11	0·12	0·15	0·49	0·87	1·57	28·6	136	72·4	86·5
Final product	0	0·02	0·05	0·07	0·35	0·49	4·31	—	620	59·5	144
Discarded supernatant	0	0	0·07	0·12	0·15	0·34	0·37	—	1·3	0·8	3·5
Albumin	0·36	0·08	0	0	0	0·44	0·71	100	2	100	3
Immediately after digestion	0·02	0·08	0·28		0·27	0·65	0·89	—	—	—	—
After heat denaturation	0	0·16	0·15		0·20	0·51	0·70	—	—	—	—
Final product	0	0·07	0	0	0·18	0·25	0·34	—	1	5·9	3
Discarded supernatant	—	—	—	—	—	—	0·08	—	—	—	—
Water-insoluble euglobulin	0	0	0·22	0·62	1·35	2·19	3·51	100	115	100	32·9
Immediately after digestion	0	0·56	0·58		0·89	2·03	3·25	93	110	87·0	30·7
After heat denaturation	0	0·12	0·14		0·36	0·62	0·57	16·2	56	48·7	98·5
Final product	0	0·11	0·10		0·43	0·64	1·52	—	136	45·4	89·5
Discarded supernatant	0	0	0	0	0	0	0	—	1·5	2·0	6·3
Water-soluble pseudoglobulin	1·06	0·67	0·92	2·24	1·31	6·20	9·89	100	660	100	66·7
Immediately after digestion	0·08	0·10	0·94	1·94	2·60	5·66	5·24	89·9	310	94·0	59·0
After heat denaturation	0	0·11	0·37	0·43	1·28	2·19	1·54	15·6	250	75·8	162
Final product	0	0·11	0·17	0·45	1·48	2·21	4·55	—	600	70·6	132
Discarded supernatant	0	0	0·06	0·15	0·21	0·42	0·18	1·82	0·5	0·3	2·8

Table 3. *The recovery of β-+T- and γ-globulins in the final products of enzyme purification of plasma and different fractions, as % of the original amount present*

Fraction	Volume digested (ml.)	Volume of final product (ml.)	Globulin (g./100 ml.) β-+T- Before digestion	β-+T- Final product	γ- Before digestion	γ- Final product	% recovery β-+T-	% recovery γ-
Plasma	50 000	4150	4·73	4·49	2·05	14·80	7·9	60·0
Salt-pptd. euglobulin	100	46	2·95	0·50	1·28	1·74	7·8	62·6
Salt-pptd. pseudoglobulin	150	27	1·19	0·44	0·37	1·92	6·7	92·5
Water-insol. euglobulin	53	10	0·35	0·15	0·55	0·64	10·5	20·7
Water-sol. pseudoglobulin	75	39	3·16	0·80	1·31	1·90	13·1	75·6

serum γ-globulin; however, the maximum increase from this cause cannot be more than 0·3 g./100 ml. and therefore cannot account for the observed increases of 1·16, 1·60 and 1·29 g./100 ml. respectively. It is possible that similar 'manufacture' of γ-globulin also took place during treatment of the other fractions but was obscured by such factors as the degree of digestion and loss during heat denaturation.

It is becoming difficult to escape the conclusion that the substances commonly designated as α-, β- and γ-globulins are not the fixed entities that they are often considered to be. They seem, rather, to be the labile resultants of the physical conditions prevailing during their preparation or examination. The variability and heterogeneity of globin fractions shown by McFarlane's (1935) ultracentrifuge experiments are an early example of this interchangeability, and the altered patterns obtained by Perlmann & Kaufman (1949) on electrophoresis of serum proteins after treatment with dilute acetic acid form another. The effect of phenol or cresol, reported by Harms (1946), in reversing the order of precipitation of albumin and globulins by ammonium sulphate is, probably, in the same category. It is possible that the somewhat large proportion of albumin in the salt-precipitated euglobulin fraction used in the present work may be due to the presence of cresol in the plasma serving as starting material; although the composition of the fractions derived from the plasma may be dependent to some extent on the presence of this cresol, the conclusion that the final digestion products are of almost constant composition is not invalidated.

That the distribution of immunological properties of an antiserum is also subject to variation is shown by the salting-out experiments of Barr *et al.* (1931). Another manifestation of the same phenomenon is afforded by the fact that the antibodies in different batches of typhoid antiserum cannot be consistently separated in any given globulin fraction by salt precipitation or by adjustment of the ionic strength of the solutions (Anderson, unpublished results).

The terms euglobulin and pseudoglobulin are even less applicable as describing entities than are α-, β- and γ-globulins, as may be seen from the electrophoretic and antitoxic 'make-up' of the preparations shown in Table 1.

It seems probable that the groupings on the protein molecules responsible for their immunological reactions and specificity are constant but that other parts of the molecule may be altered by the physical conditions in such a way that the antibodies appear to be differently distributed when the serum is fractionated or submitted to such processes as enzyme purification. The alteration in the 'carrier' part of the protein is probably also responsible for the observed changes in flocculation time, avidity and *in vivo/in vitro* ratios of the products of such manipulation.

SUMMARY

1. Electrophoretic examination shows that horse diphtheria-antitoxic plasma and euglobulin and pseudoglobulin fractions prepared from it by salt precipitation or by dialysis have quite different proportions of α-, β-, T- and γ-globulins and different distribution of antitoxin.

2. These changes of composition are at least partly due to conversion of one component into another and not only to selective precipitation or to loss.

3. Peptic digestion, followed by heat denaturation and ammonium sulphate precipitation, gives products from the original plasma and from all of the fractions with about 70% of γ-globulin and 20–30% of β-+T-globulins and containing 50–60% of the original antitoxic activity. The recovery of γ-globulin is always greater than that of β-+T-globulins and is usually much greater.

4. It is suggested that fractionation and other physical conditions affect the apparent distribution of antitoxin and also alter the properties of the 'carrier' part of the antibody molecule in such a way as to modify such characteristics as flocculation time, avidity and *in vivo/in vitro* ratio without changing specificity and antitoxic properties.

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The Effects of Salicylate and Adrenocortical Preparations Added *in vitro* upon the Glycogen Content of Liver Slices

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The injection of salicylate has been observed to cause a marked depletion of the liver-glycogen level in normal rats (Lutwak-Mann, 1942; Smith, Meade & Bornstein, 1952) and to prevent the deposition of liver glycogen induced by the injection of cortisone in adrenalectomized rats (Smith, 1952). The present paper is concerned with the effects of salicylate and adrenocortical preparations added *in vitro* on the glycogen content of rat-liver slices incubated in buffered physiological salt solutions.

EXPERIMENTAL

Preparation of tissue. Male Wistar rats, weighing 150–300 g., maintained on a diet of cubes (Medical Research Council diet 41) were used. The rats were killed by stunning, the liver was removed and slices of thickness 0·3–0·4 mm. were prepared with a scalpel, always from the same lobe. The slices as cut were placed serially in a number of piles, equal to the number of samples needed, in a Petri dish over ice. Each sample, weighing 80–100 mg., consisted of about four slices. The samples were weighed at the beginning of the experiment on a torsion balance. Samples in duplicate were used throughout and the average difference between duplicates for the glycogen estimations was $\pm 6{\cdot}3\%$.

Conditions of incubation. Two salt solutions were used: (*a*) phosphate–Ringer (Seckel, 1940): 8·0 g. NaCl, 1·0 g. $NaHCO_3$, 0·1 g. KCl and 0·1 g. $CaCl_2$, H_2O were dissolved in 1 l. of distilled water; 90 ml. of this solution were mixed with 7·0 ml. M/15 Na_2HPO_4 solution and 3·0 ml. M/15 NaH_2PO_4 solution before use; (*b*) solution containing K 110, Mg 20, Ca 10, HCO_3 40 and Cl 100 m-atoms/l. (Hastings, Teng, Nesbett & Sinex, 1952). The initial pH was 7·4 in all cases, an atmosphere of air or O_2 being used with (*a*) and 95% O_2–5% CO_2 (by vol.) with (*b*).

The samples, each with 2 ml. of medium, were placed in Warburg flasks which were attached to manometers and shaken continuously at 37°. At the end of the experiment the slices were transferred as rapidly as possible to 30% (w/v) KOH. The slices for initial values of glycogen were placed in 30% (w/v) KOH at the time the experimental samples went into the bath.

Materials. Salicylic acid (A.R.) was dissolved in the salt solutions to give final concentrations between 0·005 M and 0·02 M. 0·005 M salicylate (69 mg./100 ml.) was chosen as a suitable initial concentration because plasma salicylate concentrations of 50–70 mg./100 ml. were found in rats at the time when maximum liver-glycogen depletion occurred following the subcutaneous injection of sodium salicylate.

The adrenocortical extract used was Eschatin, free from preservative (Parke, Davis and Co.), 1 ml. of which was

equivalent to 90 g. fresh adrenals. The amount used was 0·3 ml. for each flask (0·15 ml./ml. medium), as this quantity was reported by Chiu & Needham (1950) to give a maximum effect in decreasing glycogen disappearance.

Cortisone and hydrocortisone alcohols were dissolved in Seckel's phosphate–Ringer solution to give final concentrations ranging from 30 to 60 μg./ml. medium in the Warburg flasks. During the early part of the work, the commercial suspension of cortisone acetate (Cortone acetate, Merck) was used, but a clear solution could not be obtained at dilutions of less than 1 in 200 (12·5 μg. cortisone acetate/ml.). No effect on glycogen breakdown in the liver slices was obtained with this concentration and the free cortisone alcohol, which is 14 times as soluble as the acetate (Macek, Baade, Bornn & Bacher, 1952) was used to produce the higher concentrations.

Analytical methods. Glycogen was separated by the method of Good, Kramer & Somogyi (1933), the glucose in the final stage of the estimation being measured by the method of Nelson (1944). The Eschatin was analysed for cortisone and hydrocortisone by the method of de Courcy, Bush, Gray & Lunnon (1953) and for potassium and sodium by the flame photometer.

RESULTS

The results in Table 1 show the effect of the addition of salicylate on the glycogen disappearance from liver slices incubated in Seckel's phosphate–Ringer solution in an atmosphere of air. The results are expressed as percentages of the glycogen level at the beginning of the experiment. This initial glycogen content was 1·34–5·10 g./100 g. wet tissue.

Comparison of the results by Student's t test showed that there was no significant difference between the amounts of glycogen broken down in the control experiments and in the flasks containing salicylate concentrations up to 0·02 M (276 mg./100 ml.).

When the experiments were repeated substituting the salt solution of Hastings *et al.* (1952) for Seckel's phosphate–Ringer and incubating for 60 min. under an atmosphere of 95 % O_2–5 % CO_2, it was found that the addition of 0·005 M salicylate caused a significant increase in glycogen disappearance ($t = 2·8909$, $P = 0·02$) in the slices. The results from eight rats, expressed as percentages of the initial glycogen content of the slices, were 48·2 ± 7·9 (range, 34·3–55·8) for the control and 37·4 ± 7·0 (range, 23·7–45·0) for the flasks containing 0·005 M salicylate.

As shown in Table 2, Eschatin caused a significant decrease in glycogen disappearance in the absence, but not in the presence, of 0·005 M salicylate.

Table 1. *The effect of salicylate on glycogen content of liver slices incubated aerobically*

About 100 mg. of slices were suspended in 2 ml. of Seckel's phosphate–Ringer under an atmosphere of air and incubated for 30 or 60 min. at 37° with shaking. Mean values ± standard error and ranges are given for the results, which are expressed as percentages of the initial glycogen content.

Molar concentration of salicylate	No. of rats	30 min.		60 min.	
		Control	Salicylate	Control	Salicylate
0·005	6	48·1 ± 20·0	46·8 ± 20·1	31·6 ± 18·0	31·3 ± 18·4
	Range	22·2–70·5	20·8–65·8	9·0–51·0	9·0–51·4
0·01	7	58·2 ± 19·2	51·2 ± 19·7	39·4 ± 19·9	32·3 ± 16·9
	Range	36·6–84·1	29·2–78·7	13·4–66·8	10·4–58·8
0·02	9	50·0 ± 15·1	42·7 ± 15·4	28·2 ± 14·1	21·5 ± 12·3
	Range	30·2–67·7	27·0–66·4	15·5–55·5	11·9–47·3
Statistical significance of the differences between the results for the control and salicylate groups					
0·005 M	—	$t = 0·1119$	$P = 0·9$	$t = 0·0285$	$P = 0·9$
0·01 M	—	$t = 0·6715$	$P = 0·6$	$t = 0·7169$	$P = 0·5$
0·02 M	—	$t = 1·0123$	$P = 0·4$	$t = 1·0713$	$P = 0·3$

Table 2. *The effect of Eschatin, with and without* 0·005 M *salicylate, on the glycogen content of liver slices incubated aerobically*

About 100 mg. of slices were suspended in 2 ml. of Seckel's phosphate–Ringer under an atmosphere of oxygen and incubated for 60 min. at 37° with shaking.

No. of rats	% of initial glycogen content after incubation			
	Control (*A*)	Eschatin (*B*)	Salicylate (*C*)	Eschatin + salicylate (*D*)
6	19·2 ± 9·5	32·3 ± 7·3	18·6 ± 7·0	21·4 ± 6·8
Range	12·5–29·2	22·1–45·4	8·7–28·9	12·2–34·3

Statistical significance of differences between results

For groups *A* and *B*: $t = 2·6974$, $P = 0·02$; for groups *C* and *D*: $t = 0·6541$, $P = 0·5$.

Table 3. *The effect of Eschatin, cortisone and hydrocortisone on glycogen content of liver slices incubated aerobically*

About 100 mg. of slices were incubated in 2 ml. Seckel's phosphate–Ringer under an atmosphere of oxygen for 60 min. at 37° with shaking. Cortisone and hydrocortisone present in concentrations of either 30 or 60 μg./ml. medium.

	% of initial glycogen content after incubation					
			Cortisone		Hydrocortisone	
No. of rats	Control	Eschatin	(30 μg./ml.)	(60 μg./ml.)	(30 μg./ml.)	(60 μg./ml.)
7	31·0±7·9	49·6±11·2	29·8±8·3	29·4±7·7	28·5±7·0	26·7±6·2
Range	18–40	29–61	16–42	17–38	16–35	16–34

Statistical significance of differences between results

For control and Eschatin: $t = 3{\cdot}5876$, $P = 0{\cdot}01$; for control and cortisone, and for control and hydrocortisone: $P > 0{\cdot}3$ in all cases.

As shown in Table 3, although Eschatin caused a significant decrease in glycogen disappearance, cortisone and hydrocortisone in concentrations of 30 and 60 μg./ml. medium had no effect. Analysis of the Eschatin for adrenocortical steroids by the method of de Courcy *et al.* (1953) showed that the extract contained approx. 100 μg./ml. of cortisone and 50 μg./ml. of hydrocortisone. 0·15 ml. Eschatin/ml. medium was therefore equivalent to approx. 16 and 8 μg. respectively of the two steroids/ml. of medium in the Warburg flasks. The Eschatin contained 0·8 mg./100 ml. of K and 370 mg./100 ml. of Na.

DISCUSSION

When liver slices are incubated in buffered physiological salt solutions in air or oxygen the observed degree of glycogen disappearance is the resultant of simultaneous breakdown and synthesis. The importance of the cationic composition of the incubation medium has been emphasized by Hastings *et al.* (1952), who demonstrated that the sodium/potassium ratio in particular affected the amount of glycogen formation by rat-liver slices. A high sodium/potassium ratio decreased the amount of new glycogen formed whereas a low sodium/potassium ratio produced the reverse effect.

The two buffered salt solutions used in the present work differed in that Seckel's phosphate–Ringer contained mainly sodium salts with a very small proportion of potassium salts, whereas the solution of Hastings *et al.* contained no sodium and a high proportion of potassium. When the liver slices were incubated in these two solutions, more glycogen disappeared in the former than in the latter, the average results after 60 min. incubation, expressed as percentages of the initial glycogen content, being 33·0 and 48·2 respectively. If the difference was due to greater glycogen formation occurring in the slices incubated in the solution of Hastings *et al.*, then the observed effect of added salicylate in causing more glycogen disappearance in this solution, but not in Seckel's phosphate–Ringer, may be caused by an interference with the processes concerned with the synthesis rather than the breakdown of glycogen. This *in vitro* effect was produced at salicylate concentrations similar to those observed in the blood of normal rats showing marked depletion of liver glycogen after injection of salicylate. Interference with glycogen synthesis in the liver may therefore contribute to this depletion *in vivo*.

Seckel (1940) reported that addition of adrenocortical extract (Eschatin, free from preservative) to phosphate–Ringer solution in which liver slices were incubated caused a decrease in glycogen disappearance. His observation was confirmed by Chiu & Needham (1950), who showed that the Eschatin increased the formation, rather than decreased the breakdown, of glycogen. The present results again confirm the work of Seckel and also show that the addition of 0·005 M salicylate inhibits the increased glycogenesis induced by the adrenocortical extract. The possibility was considered that Eschatin might contain a high proportion of potassium salts and that the addition of 0·3 ml. to 2 ml. of Seckel's phosphate–Ringer might increase the potassium/sodium ratio in the final incubation medium, thus causing increased glycogenesis. However, the extract was found to contain only a small concentration of potassium and a relatively large amount of sodium and this explanation was therefore not valid.

The next series of experiments was designed to determine if the antagonism between salicylate and the adrenal-cortex extract could be extended to pure adrenocortical steroids, because Chiu (1950) claimed that certain steroids, including cortisone, acted similarly to Eschatin *in vitro*. However, no effect of cortisone or hydrocortisone, in concentrations greater than those employed by Chiu (1950) and present in the Eschatin, on glycogen disappearance in rat-liver slices, could be demonstrated. Verzár (1951) has also reported that he did

not succeed in obtaining significant glycogen production with rat-liver slices incubated with corticosteroids *in vitro* in the presence of glucose or pyruvate as substrates. The present results indicate first, that the well-marked *in vitro* action of Eschatin cannot be attributed to its content of cortisone or hydrocortisone and, secondly, that passage of cortisone through the body is necessary for the injection of the hormone to produce liver-glycogen deposition in adrenalectomized rats. The *in vitro* antagonism of salicylate and Eschatin observed in the present work and the effect of salicylate in preventing the deposition of liver glycogen induced by cortisone in the adrenalectomized rats (Smith, 1952) therefore cannot be explained on a common basis.

SUMMARY

1. The effects of the addition of salicylate, adrenocortical extract, cortisone and hydrocortisone on the glycogen content of rat-liver slices incubated in buffered physiological salt solutions have been investigated.

2. Salicylate caused a significant increase in the glycogen disappearance in liver slices incubated in a medium containing a high concentration of potassium and no sodium, but not in a medium containing a high proportion of sodium.

3. Adrenocortical extract (Eschatin, free from preservative) but not cortisone or hydrocortisone, produced a significant decrease in the glycogen disappearance and this effect was inhibited by the presence of salicylate.

4. The implications of these effects are discussed.

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The Action of Polylysine on the Conversion of Fibrinogen into Fibrin by Coagulase Thrombin

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It has been shown previously that polylysine, a synthetic basic water-soluble poly-α-amino acid, retards blood coagulation and this was shown to be due to interference with the formation of thrombin from prothrombin (de Vries, Schwager & Katchalski, 1951). The present study was undertaken to investigate a possible effect of polylysine on the clotting of plasma by staphylocoagulase. Polylysine was found to accelerate this clotting reaction.

The coagulation of plasma by staphylocoagulase proceeds in two stages: (*a*) staphylocoagulase reacts with a coagulase-reacting factor present in plasma to form coagulase thrombin, (*b*) coagulase thrombin converts fibrinogen into fibrin (Miale, 1949; Tager, 1948). The action of polylysine on the clotting of fibrinogen in the presence of staphylocoagulase and coagulase-reacting factor was therefore investigated.

EXPERIMENTAL

Materials

Staphylocoagulase. This was obtained by the method of Duthie & Lorenz (1952) from a strain of *Staphylococcus aureus*, Newman (NCTC 8178), grown in broth or in brain–heart infusion for 24 hr. The organisms were removed by centrifuging in a Sorvall centrifuge for 15 min. at 12000 rev./min. at 4°.

Coagulase-reacting factor. This was obtained from pooled oxalated human plasma according to the method of Kaplan & Spink (1948), and stored in 0·9% NaCl at 4°.

Human plasma. Venous blood was mixed with $\frac{1}{9}$ vol. of 0·1 M sodium oxalate and centrifuged for 15 min. at 2000 rev./min.

Bovine plasma fraction I. This was obtained from Armour & Co., Chicago, U.S.A. It contained 45% protein, 45% of which was clottable by thrombin.

Purified fibrinogen. This was obtained from fraction I by the procedure of Laki (1951) and contained 91% of thrombin-clottable protein.

Poly-L-lysine hydrobromide (with an average degree of polymerization n 36). This was prepared according to the method of Katchalski, Grossfeld & Frankel (1948). Solutions were prepared in 0·9% NaCl.

Poly-L-aspartic acid (average degree of polymerization n 130). This was prepared by the method of Berger & Katchalski (1951) and dissolved in NaOH; the pH was adjusted to 7·2.

Protamine sulphate. This was purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio, U.S.A.).

Clotting times. These were determined in test tubes (7 mm. int. diam.) in a water bath at 37°. The first appearance of macroscopically visible fibrin strands was taken as the moment of clotting. All solutions were adjusted to pH 7·2 with 0·1 N-NaOH or 0·1 N-HCl, and brought to 37° before the performance of the coagulation tests.

Evaluation of staphylocoagulase activity. One unit of staphylocoagulase was defined as the amount inducing the appearance of fibrin strands at the end of 2 hr. at 37°, in a mixture containing 0·2 ml. staphylocoagulase solution and 0·2 ml. pooled standard oxalated human plasma diluted tenfold with 0·9% NaCl, adjusted to pH 7·2. A regression line was constructed from clotting times obtained with various dilutions of the partially purified staphylocoagulase preparation. The amount of staphylocoagulase in the various preparations was determined from the values of the regression line corresponding to the measured clotting times. A purified preparation contained 6×10^{-5} mg. protein N per unit of staphylocoagulase.

RESULTS

Effect of polylysine on the clotting of fraction I in the simultaneous presence of purified staphylocoagulase and coagulase-reacting factor

Fig. 1 shows that polylysine accelerates the clotting rate of fraction I in the simultaneous presence of staphylocoagulase and coagulase-reacting factor, and that the acceleration increases with the amount of polylysine. A similar effect was observed with protamine, which accelerated the clotting of the above system to approximately the same extent.

Fig. 2 gives the times of clotting of purified fibrinogen by staphylocoagulase and coagulase-reacting factor in the presence of a constant concentration of polylysine at different concentrations of fibrinogen. Polylysine shows a marked accelerating effect on the clotting reaction throughout the tested range of fibrinogen concentrations.

The accelerating action of polylysine on the clotting of fibrinogen was also observed in crude systems consisting of plasma supplemented either by staphylocoagulase or by the supernatant fluid of the staphylococcal culture.

Inhibition of the accelerating action of polylysine by polyaspartic acid. Table 1 shows that poly-L-aspartic acid inhibits the accelerating action of polylysine on the clotting of the staphylocoagulase–coagulase-reacting factor–fibrinogen mixture. Polyaspartic acid by itself had no influence on the clotting time in the concentration range used.

Identification of the reaction step affected by polylysine

It has been pointed out that the coagulation of blood by staphylocoagulase proceeds in two stages; it was, therefore, of interest to investigate which of the reactions involved is accelerated by polylysine.

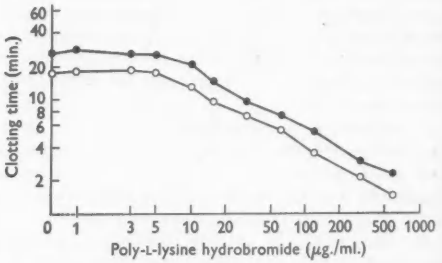

Fig. 1. Effect of various concentrations of polylysine on the clotting of fraction I by purified staphylocoagulase and coagulase-reacting factor. The clotting mixtures contained: 0·1 ml. purified staphylocoagulase, 0·1 ml. coagulase-reacting factor, 0·1 ml. poly-L-lysine hydrobromide solution and 0·3 ml. fraction I (0·2%). ●, 47 staphylocoagulase units/ml. reaction mixture; ○, 94 staphylocoagulase units/ml. reaction mixture.

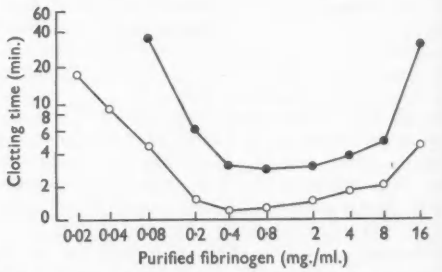

Fig. 2. Effect of the polylysine on the clotting of purified fibrinogen at various concentrations by purified staphylocoagulase and coagulase-reacting factor. The clotting mixtures contained: 0·1 ml. purified staphylocoagulase (750 units/ml.), 0·1 ml. coagulase-reacting factor, 0·1 ml. saline (●) or polylysine hydrobromide solution (2 mg./ml.) (○) and 0·3 ml. purified fibrinogen solution in 0·9% NaCl.

Action of polylysine on the clotting of fraction I by coagulase thrombin. Coagulase thrombin was obtained by incubating equal volumes of purified staphylocoagulase (498 units/ml.) and coagulase-reacting factor for 2 hr. at 37°. The incubation mixture was filtered twice through a Seitz filter. This filtrate contained some residual staphylocoagulase but no free coagulase-reacting factor

The accelerating action of polylysine on the clotting of fibrinogen by the system containing the preformed coagulase thrombin thus prepared is demonstrated in Table 2. Control experiments showed that staphylocoagulase by itself does not clot fibrinogen in the presence of polylysine.

Lack of action of polylysine on the formation of coagulase thrombin. In a study of a possible effect of polylysine on the first stage of the clotting reaction, i.e. staphylocoagulase + coagulase-reacting factor → coagulase thrombin, use was made of the property of polyaspartic acid to inhibit the action of polylysine. The experiment was performed in two steps. In the first, staphylocoagulase was incubated for different periods with coagulase-reacting factor in the presence or absence of polylysine and polyaspartic acid ('initial incubation mixture'). In the second step, samples taken from the 'initial incubation mixture' were added to fraction I to which polylysine, polyaspartic acid or both had been added ('final clotting mixture'). The results summarized in Table 3 show that in the absence of polyaspartic acid, polylysine accelerated the clotting when present in the initial incubation mixture (Expt. 2) or in the final clotting mixture (Expt. 3). When polylysine was present in the initial incubation mixture and polyaspartic acid in the final clotting mixture (Expt. 6), no acceleration of clotting was noticed. It may thus be concluded that the formation of coagulase thrombin is unaffected by polylysine.

Table 1. *Inhibiting effect of polyaspartic acid on the accelerating action of polylysine in a staphylocoagulase clotting system*

The reaction mixtures consisted of 0·3 ml. fraction I (0·2%), 0·1 ml. coagulase-reacting factor and the other components as tabulated, all in a final volume of 0·6 ml. Staphylocoagulase, 83 units/ml. in Expt. 1, 31 units/ml. in Expt. 2.

Poly-L-lysine HBr (*n** 36) (μg./ml.)	Poly-L-aspartic acid (*n** 130) (μg./ml.)	Clotting time (min.) Expt. 1	Expt. 2
—	—	8	15
125	—	2	3
125	125	6	13
125	150	7·5	14
125	250	8	14
125	500	8	15
—	500	8	15

* Degree of polymerization.

Table 2. *Accelerating effect of polylysine on the clotting of fraction I by coagulase thrombin*

The clotting mixtures contained: 0·1 ml. fraction I (0·4%), 0·1 ml. polylysine solution and 0·2 ml. coagulase thrombin solution. The latter was prepared by incubating equal volumes of staphylocoagulase (498 units/ml.) with coagulase-reacting factor for 2 hr. at 37° and diluting three times with saline.

Poly-L-lysine HBr (*n** 36) (μg./ml.)	Clotting time (min.)
0	33
5	30
12·5	25
25	20
50	14
75	7
150	4
250	3
500	2

* Degree of polymerization.

Table 3. *Absence of effect of polylysine on the formation of coagulase thrombin*

The initial incubation mixture consisted of: 1 vol. staphylocoagulase solution (1365 units/ml.), 1 vol. coagulase-reacting factor solution containing the desired amount of polyamino acid. The mixture was made up to 3 vol. with saline. The final clotting mixture consisted of: 0·3 ml. fraction I (0·2%) containing the desired amount of polyamino acid and a 0·2 ml. sample withdrawn from the initial incubation mixture at the time intervals specified. The volume of this mixture was made up to 0·6 ml. with saline.

Expt. no.	Polyamino acid added to: Initial incubation mixture — Polylysine HBr (μg./ml.)	Initial incubation mixture — Polyaspartic acid (μg./ml.)	Final clotting mixture — Polylysine HBr (μg./ml.)	Final clotting mixture — Polyaspartic acid (μg./ml.)	Incubation times of initial incubation mixtures (min.) 0	10	30	60	120	180
					Clotting times of final clotting mixtures (min.)					
1	—	—	—	—	23	20	17	18	25	23
2	411	—	—	—	2·5	2·5	3	3	3·5	4
3	—	—	138	—	3·5	3	3·5	3·5	3·5	4
4	—	4110	—	—	22	20	17	18	20	24
5	411	4110	—	—	21	20	16	18	24	24
6	411	—	—	1380	21	20	16	19	25	23
7	—	4110	138	—	21	19	16·5	18	25	24
8	—	—	138	1380	21	19·5	17	18	24	22
9	—	—	—	1380	22	20	17·5	18	25	23

DISCUSSION

Polylysine has been shown to possess various biological activities. It is bacteriostatic (Katchalski, Bichowski-Slomnitzki & Volcani, 1953) and virostatic (Stahmann, Graf, Patterson, Walker & Watson, 1951); it inhibits thromboplastic activity (de Vries *et al.* 1951), fibrinolysis (Ginsburg, de Vries & Katchalski, 1952), the proteolytic activity of pepsin (Katchalski, Berger & Neumann, 1954) and the clearing action of heparin on alimentary lipemia (Stein, de Vries, Wislicki & Katchalski, 1954).

In all the above-mentioned cases polylysine acts as a retarder of biological and enzymic processes. The effect reported here on the staphylocoagulase clotting reaction demonstrates that polylysine can also have an accelerating action in a specific biological system. This accelerating action of the basic peptide has been shown to affect the second stage of the staphylocoagulase clotting system, i.e. the clotting of fibrinogen by coagulase thrombin. In preliminary experiments it has also been observed that polylysine accelerates the clotting of fibrinogen by thrombin.

The mechanism of this acceleration is unknown. The following possibilities should be considered: (*a*) polylysine inactivates a coagulase thrombin inhibitor, (*b*) as a result of the interaction between polylysine and fibrinogen, a complex is formed which is attacked by the clotting agent more readily than fibrinogen itself, (*c*) the clotting time is shortened as the result of the ability of the highly charged threadlike polylysine molecules to form a link between the clotting agent and the fibrinogen; the local concentration of the clotting agent surrounding the fibrinogen molecules is thus increased and the clotting reaction enhanced.

The fact that polylysine and protamine exert an accelerating activity on the staphylocoagulase clotting system, whereas polyaspartic acid shows no effect, suggests that this ability is associated with the polycationic nature of the effective peptides.

SUMMARY

1. Poly-L-lysine accelerates the conversion of fibrinogen into fibrin in the simultaneous presence of staphylocoagulase and coagulase-reacting factor.

2. The clot-promoting activity of polylysine in the above system is due to the accelerating action of polylysine on the clotting of fibrinogen by coagulase thrombin.

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Glucuronide Metabolism in Plants

2. THE ISOLATION OF FLAVONE GLUCOSIDURONIC ACIDS FROM PLANTS*

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The flavone glucosiduronic acids baicalin and scutellarin are known to occur in plants of the genus *Scutellaria*. Mölisch & Goldschmiedt (1901) suggested, on qualitative histological evidence, that scutellarin, or a very similar compound, exists in plants of other genera. Baicalin has been isolated from the root of *Scutellaria baicalensis* (Shibata, Iwata & Nakamura, 1923), where it forms a considerable proportion (10–20 %) of the dry weight of tissue. It was also reported to occur in the foliage of *Scutellaria columnae* (Charaux & Rabaté, 1940*b*). Scutellarin was originally isolated from the leaves of *Scutellaria altissima* (Mölisch & Goldschmiedt, 1901) and has since been prepared from the foliage

* For Paper 1 of this series, see Levvy (1954).

of several other species, e.g. *S. baicalensis* and *Scutellaria indica* (Shibata *et al.* 1923), *Scutellaria scordifolia* (Miwa, 1932) and from the leaves of *Centaurea scabiosa* ('greater knapweed') (Charaux & Rabaté, 1940*a*).

The aglycones, baicalein and scutellarein, of these glucosiduronic acids are closely related, and were found to be identical with 5:6:7-trihydroxyflavone and 5:6:7:4′-tetrahydroxyflavone, respectively (Shibata *et al.* 1923; Bargellini, 1915). The proposed structures of these flavones, originally synthesized by Bargellini (1915, 1919), were subsequently questioned (for review, see Plant, 1931), but have now been confirmed by less equivocal syntheses (Sastri & Seshadri, 1946). From indirect evidence, Shibata & Hattori (1930) have suggested that the point of conjugation is at the hydroxy group in position C-7 of the flavone nucleus in each case.

The purpose of this investigation was to isolate baicalin and scutellarin as possible substrates for the enzymes baicalinase (Miwa, 1932) and animal-tissue β-glucuronidase, and for comparison with other glucosiduronic acids which might later be isolated from field crops (see Marsh & Levvy, 1953). *S. baicalensis* is a native of the Far East, where its root has, or had, some use in pharmacy, but it cannot readily be cultivated in quantity in this country. An examination of *Scutellaria galericulata* ('common skullcap'), indigenous to Britain, was therefore made; its foliage yielded a previously unknown glucosiduronic acid of chrysin (5:7-dihydroxyflavone), which closely resembled baicalin in many respects. The acid in this compound was identified after enzymic hydrolysis with animal-tissue β-glucuronidase, and the aglycone after acid hydrolysis. From *S. columnae*, which has established itself locally in England, was isolated not baicalin (see above) but scutellarin, which was also prepared from *C. scabiosa*, and from *Scutellaria tournefortii*, a new source. This last plant is easy to cultivate in Britain.

EXPERIMENTAL AND RESULTS

Materials. *S. galericulata* and *C. scabiosa* were collected in Bedfordshire during the summer of 1953 by Dr J. G. Dony. *S. columnae* was collected in Somerset in May 1954 by Mr I. W. Evans. *S. tournefortii* was cultivated in Aberdeenshire from seed presented by the Royal Botanic Gardens, Kew. Authentic samples of baicalin, baicalein and chrysin were given by Professor S. Hattori, Tokio University.

Methods. The mouse-liver β-glucuronidase used for the enzymic hydrolysis of chrysin glucosiduronic acid was prepared according to the procedure of Levvy & Marsh (1952) for use in reducing-sugar measurements. Determinations of C, H, and of the optical rotation of chrysin glucosiduronic acid were made by Weiler and Strauss, Oxford. Spectral absorption was measured with a Beckman Model DU quartz spectrophotometer. All melting points are corrected, unless otherwise stated.

Isolation of chrysin glucosiduronic acid and scutellarin

The method of extraction was in general similar to those employed by previous workers for the isolation of flavone glucosiduronic acids from plants. The fresh or air-dried material was boiled under reflux with water (10 ml./g. fresh or dried leaf) for 2 hr., then strained through surgical gauze. Smaller particles were removed by centrifuging at 1500 *g* for 15 min. After the supernatant had been concentrated *in vacuo* to a suitable volume, conc. HCl (0·1 vol.) was added to the hot solution to precipitate the crude flavone glycosides, and after 12 hr. at 0° these were filtered off and washed with water. The crude products were then powdered under cold methanol and filtered; this removed much colouring matter. The residues were extracted with methanol in a 'hot Soxhlet' apparatus for up to 48 hr. until no further solid was extracted. During this period the glucosiduronic acids crystallized from the boiling solvent. The products were filtered off after 24 hr. at 0° and recrystallized several times from 95% (v/v) aqueous methanol.

Chrysin glucosiduronic acid

From the fresh leaf of *S. galericulata*, *chrysin glucosiduronic acid* was isolated (2·6% of the dry weight of leaf, after one recrystallization from 95% aqueous methanol), but no scutellarin, the presence of which had been suggested by Mölisch & Goldschmiedt (1901) from histological observations. (Found on product dried at 140° *in vacuo*: C, 58·6; H, 4·2. $C_{21}H_{18}O_{10}$ requires C, 58·6; H, 4·2%.) The identification of chrysin and glucuronic acid in this compound is described below.

Properties. Chrysin glucosiduronic acid crystallized from aqueous methanol in fine yellow needles, m.p. 225–226° (decomp.). The loss in weight on drying at 140° *in vacuo* was 3·1%; the anhydrous product had $[\alpha]_D^{21} - 112 \pm 2°$ in 50% (v/v) aqueous pyridine (*c*, 1·2). It was quite soluble in hot acetic acid, difficultly soluble in boiling methanol, ethanol and acetone (about 0·1%), and almost insoluble in water, ether and benzene. It was easily soluble in aqueous alkalis (saturated solution about 0·05M), giving yellow solutions which became greenish on standing.

The addition of $FeCl_3$ solution to an ethanolic solution gave a greenish coloration, red by transmitted light. The addition of lead acetate solution to an aqueous pyridine solution gave a yellow precipitate of the lead salt; addition of a solution of barium acetate gave a yellow colour, and calcium chloride an orange colour, but no precipitates, as distinct from the properties of baicalin as described by Shibata *et al.* (1923). The reduction of an aqueous ethanolic solution with magnesium and HCl gave a deep yellow coloration, but no precipitate.

Chrysin glucosiduronic acid slowly reduced Fehling's solution, but rapidly reduced it after hydrolysis with 2N-HCl at 100° for 30 min. A strongly positive reaction for uronic acid was obtained with Tollens' naphthoresorcinol reagent.

The general properties of chrysin glucosiduronic acid closely resembled those of baicalin, as described by Shibata *et al.* (1923), and, indeed, no apparent depression of the melting point was observed on admixture with an authentic sample of baicalin, m.p. 222°, prepared from *S. baicalensis* root. Chrysin glucosiduronic acid and baicalin can be discriminated by their u.v. absorption spectra, as described below, provided both compounds are available.

Identification of glucuronic acid after enzymic hydrolysis of chrysin glucosiduronic acid

A sample of chrysin glucosiduronic acid was supplied to Dr G. A. Levvy and was found to be readily hydrolysed by mouse-liver β-glucuronidase (Levvy, 1954). From a previous investigation of the specificity of this enzyme (Levvy & Marsh, 1952) it could be concluded that the compound obtained from *S. galericulata* leaf was therefore a β-D-glucopyranosiduronic acid, since the enzyme is specific for the hydrolysis of this group. It was desirable to verify this assumption, however, by isolation of derivatives of glucuronic acid after the enzymic hydrolysis. The method employed was similar to that of Levvy (1948).

An aqueous solution of chrysin glucosiduronic acid (0·05M; 20 ml.) in 0·1M acetic acid—NaOH buffer at pH 5·2 was incubated with mouse-liver β-glucuronidase (5 ml.) containing a total of 4500 'glucuronidase units' (G.U.) where 1 G.U. liberates 1 μg. phenolphthalein in 1 hr. at 38° from 1·25 mM phenolphthalein glucuronide, in the same buffer.

After incubation at 38° for 48 hr., a yellow precipitate (chrysin) had formed and was removed by centrifuging at 1500 *g* for 15 min. The supernatant was then dialysed into water (200 ml.) at 3° for 48 hr. Acidification of the dialysate with 3N-H_2SO_4 to pH 2 precipitated unchanged substrate and chrysin, which were removed by filtration. Final traces of flavone compounds were then removed by extracting the filtrate with ethyl acetate (5 × 30 ml.), in which freshly acidified glucuronic acid has a negligible solubility. The aqueous solution was then shaken with $BaCO_3$ to remove sulphate, and filtered. A small sample of the filtrate was removed, and the free glucuronic acid content determined by titration with ceric sulphate, according to the procedure of Levvy (1946). When corrected for the free glucuronic acid remaining in the dialysis sac, it was calculated that 75 mg. glucuronic acid were liberated, estimated as reducing sugar. This corresponded to 39% hydrolysis of the total substrate. A control experiment showed that in the absence of enzyme no free-reducing sugar was liberated.

The aqueous solution, calculated to contain 63 mg. glucuronic acid, was evaporated *in vacuo* to about 2 ml. and oxidized to D-glucosaccharic acid, followed by coupling with *o*-phenylenediamine, according to the procedure of Lohmar, Dimler, Moore & Link (1942) as modified by Levvy (1948). This yielded 28 mg. of the dibenziminazole derivative (24% of theoretical from the glucuronic acid content), m.p. 236–237° (decomp.). From this was prepared the dihydrochloride, m.p. 265–267° (decomp.) and dipicrate, m.p. 210–211° (decomp.) with change of form at 140–142°. Authentic specimens of these compounds were prepared from D-glucurone (180 mg.) by oxidation and subsequent coupling using the same procedure. This yielded 110 mg. (30% of theoretical) of the dibenziminazole derivative of D-glucosaccharic acid, m.p. 240° (decomp.), from which were obtained the dihydrochloride, m.p. 267–268° (decomp.), $[\alpha]_D^{21} + 52{\cdot}0 \pm 0{\cdot}5°$ in water (*c*, 2), and the dipicrate m.p. 212° (decomp.) with change of form at 142°. These data are in good agreement with those obtained by Levvy (1948).

None of the derivatives obtained from the products of enzymic hydrolysis when admixed with the corresponding authentic specimens produced any depression of the melting points.

The washed and dried flavone precipitates obtained in this experiment were combined and boiled under reflux with ether (200 ml.). The mixture was filtered and the filtrate evaporated to dryness and recrystallized from 80% (v/v) aqueous ethanol to yield chrysin (58 mg.; 59% of theoretical), m.p. 270–272° (uncorr.). The ether-insoluble residue was recrystallized from 95% (v/v) aqueous methanol, yielding chrysin glucosiduronic acid (128 mg.; 30% of starting material), m.p. 224° (decomp.).

This experiment gave satisfactory proof that the sugar liberated by hydrolysis of the glycoside from *S. galericulata* leaf was D-glucuronic acid.

Scutellarin

From C. scabiosa. Scutellarin was isolated from the foliage (2·4% of the dry weight of leaf, after two recrystallizations). The product, even when crude, had no melting point below 300°, but darkened slowly above 230°, whereas Charaux & Rabaté (1940*a*) stated that when isolated from this source it had m.p. 205–230°. The present finding agrees with the earlier workers (Mölisch & Goldschmiedt, 1901; Shibata *et al.* 1923), who obtained no definite melting point below 300°. (Found on product dried at 130° *in vacuo*: C, 54·4; H, 4·5. Calc. for $C_{21}H_{18}O_{12}$: C, 54·5; H, 3·9%.)

From S. tournefortii. The freshly cut leaf yielded scutellarin (3·3% of the dry weight of leaf, after one recrystallization). The impure product melted at about 195°, above which temperature it apparently resolidified; this effect was due to the presence of a more soluble impurity, for after several further recrystallizations the product darkened slowly above 230°, but did not melt below 300° (see above). (Found on product dried at 130° *in vacuo*: C, 54·2, H, 4·1. Calc. for $C_{21}H_{18}O_{12}$: C, 54·5; H, 3·9%.)

From S. columnae. The foliage of this plant yielded scutellarin (4·5% of the dry weight of leaf), but no baicalin (cf. Charaux & Rabaté, 1940*b*). The product slowly darkened above 220°, but did not melt below 300°.

Preparation of the aglycones by acid hydrolysis of the glucosiduronic acids

The finely powdered anhydrous glucosiduronic acids (150 mg.) were hydrolysed by boiling with a mixture of acetic acid (15 ml.) and dil. H_2SO_4 (10%, w/v; 15 ml.) under reflux for 5 hr. (Charaux & Rabaté, 1940*a*). The glycosides slowly passed into solution as the reaction proceeded. The solutions were then concentrated *in vacuo* at 45° to remove acetic acid, and diluted with water (40 ml.) to precipitate the aglycones. The mixtures were then extracted thrice with ether (total 60 ml.) and the ethereal solutions washed with water and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residues were recrystallized from aqueous ethanol (80%, v/v). The yields of the crude products were almost quantitative.

The acetyl derivatives of the aglycones were prepared by heating with anhydrous sodium acetate and acetic anhydride in the usual way, and were recrystallized from ethanol.

Chrysin. Hydrolysis of the glucosiduronic acid isolated from *S. galericulata* yielded chrysin, m.p. 268–269° (uncorr.). (Found on product dried at 130°: C, 70·4; H, 4·7. Calc. for $C_{15}H_{10}O_4$: C, 70·8; H, 4·0%.) There was no depression in the melting point on admixture with an authentic sample of chrysin, m.p. 275° (uncorr.), nor, however, with an authentic

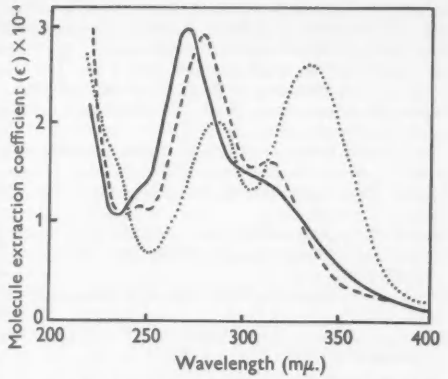

Fig. 1. The ultraviolet absorption spectra of 0·05 mM chrysin glucosiduronic acid (——), baicalin (– – –) and scutellarin (. . . . .) in ethanol.

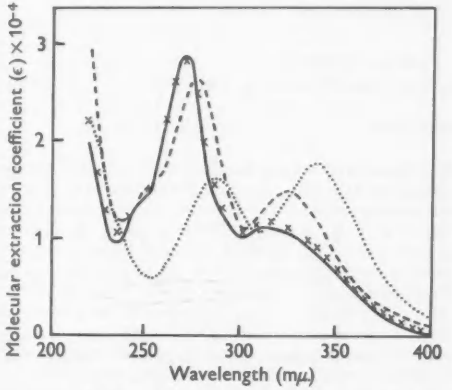

Fig. 2. The ultraviolet absorption spectra of 0·05 mM chrysin, baicalein and scutellarein in ethanol; ——, chrysin from *S. galericulata*; ×—×, authentic chrysin; – – –, baicalein; . . ., scutellarein.

sample of baicalein, m.p. 262° (uncorr.) (decomp.) derived from *S. baicalensis* root. These flavones could, however, be distinguished by their u.v. absorption spectra (see below) when both were available.

Acetylation of chrysin (from *S. galericulata*) yielded chrysin diacetate as colourless needles, m.p. 190–192°. (Found on product dried at 130°: C, 66·8; H, 4·2; CH_3CO, 24·8. Calc. for $C_{19}H_{14}O_6$: C, 67·4; H, 4·1; CH_3CO; 25·4%.) There was no depression of the melting point on admixture with an authentic sample of chrysin diacetate, m.p. 189–191°, but when mixed with an authentic sample of baicalein triacetate, m.p. 188–190°, the melting point was 163–168°. The acetates of the aglycones provide the most satisfactory method for distinguishing chrysin glucosiduronic acid from baicalin.

Scutellarein. The products obtained on hydrolysis of scutellarin from *C. scabiosa*, *S. tournefortii* and *S. columnae* did not melt below 300°. Acetylation of scutellarein gave the tetraacetate as colourless prisms. The acetate derived from *C. scabiosa* had m.p. 237° (found on product dried at 120°: C, 60·6; H, 4·1; CH_3CO, 37·9%), that from *S. tournefortii* had m.p. 235–237° (found on product dried at 120°: C, 60·9; H, 4·2; CH_3CO, 38·7. Calc. for $C_{23}H_{18}O_{10}$: C, 60·8; H, 4·0; CH_3CO, 37·9%), and that from *S. columnae* had m.p. 235°. There was no depression of the melting points on admixture of any of the scutellarein acetates from the different sources.

Table 1. *Ultraviolet absorption maxima and minima of ethanolic solutions of chrysin glucosiduronic acid, baicalin, scutellarin and their aglycones*

~ Denotes an inflexion.

Compound	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$	$\lambda_{min.}$ (mμ.)	$\epsilon_{min.}$
Chrysin glucosiduronic acid	270	29 600	—	—
	~306	14 400	235	10 300
Baicalin	246	11 800	237	10 900
	279	30 000	250	11 400
	314	16 400	305	15 900
Scutellarin	285	20 000	252	6 400
	335	26 100	305	12 300
Chrysin	270	28 500	235	10 400
	318	11 900	302	10 600
Baicalein	276	26 600	238	11 700
	324	15 100	302	11 000
Scutellarein	286	16 600	252	6 300
	339	18 300	310	10 700

Ultraviolet absorption spectra

A sensitive method of distinguishing between chemically similar flavonoid compounds is by a comparison of their absorptions in the ultraviolet (see Hattori, 1932). The spectra of ethanolic solutions of chrysin glucosiduronic acid, baicalin and scutellarin are shown in Fig. 1, and those of chrysin, baicalein and scutellarein in Fig. 2. The spectrum of chrysin obtained from *S. galericulata* corresponded closely with that of an authentic specimen of chrysin, and the spectra of specimens of scutellarin and scutellarein from *C. scabiosa*, *S. tournefortii* and *S. columnae* were found to be identical.

The absorption maxima and minima of these compounds are given in Table 1. An increase in the number of hydroxyl groups on the flavone nucleus caused a general bathychromic shift of the spectra of both the glucosiduronic acids and of the aglycones; the absorption maximum at the lower wavelength was simultaneously weakened, and that at the higher wavelength strengthened. These general observations confirm the work of Hattori (1932).

SUMMARY

1. Chrysin glucosiduronic acid was isolated from the leaves of *Scutellaria galericulata*, the glucuronic acid residue being identified after enzymic hydrolysis, and the aglycone after acid hydrolysis.

2. Scutellarin was isolated from the foliage of *S. tournefortii* and *S. columnae*, the products being identical with that obtained from the foliage of *Centaurea scabiosa*.

3. The ultraviolet absorption spectra of the flavone glucosiduronic acids and of their aglycones have been compared.

The author wishes to thank Dr G. A. Levvy for his interest in this work, and Dr J. G. Dony and Mr I. W. Evans for their willing co-operation in obtaining plant material. He is indebted to the Royal Botanic Gardens, Kew, for presenting seeds of *S. tournefortii*.

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The Amino Acid Composition of Wool

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When the present work was started early in 1953 no complete amino acid analysis of a single wool sample had been published, although Graham, Waitkoff & Hier (1949) had estimated fourteen amino acid constituents of a wool hydrolysate, chiefly by microbiological methods. While this paper was in preparation, however, our attention was drawn to a paper by Simmonds (1954), who has completed the amino acid analysis of an Australian 64's quality wool. The wool sample whose analysis is reported here was of the same quality, and it is of interest, therefore, to compare the two analyses. For a survey of amino acid analyses of wool, previously published in the literature, reference should be made to Simmonds (1954).

EXPERIMENTAL

Chromatographic separations and estimations of amino acids

All the columns used for starch chromatography were of 0·9 cm. diameter and 30 cm. length and were prepared according to the directions of Stein & Moore (1948) from starch manufactured by Gordon Slater Ltd., Manchester. Six different types of chromatograms were employed in the analysis of wool hydrolysates. Leucine, isoleucine, phenylalanine and methionine were separated on starch columns eluted with benzyl alcohol–*n*-butanol–water (1:1:0·280, by vol.). The volume of water in the mixture was smaller than that used by Stein & Moore (1948) because the columns were run at 18°. The columns were loaded with 3·5 mg. of hydrolysate and 0·5 ml. fractions were collected for the estimations of leucine, phenylalanine and isoleucine, but for the accurate estimation of methionine, which occurs to the extent of 0·5% in the hydrolysate, 10 mg. loads were applied and 1·5 ml. fractions collected. Thiodiglycol was added to the eluting solvent for the methionine estimations. This chromatogram is illustrated in Fig. 1 and referred to hereafter as starch I. Valine and tyrosine were separated from 2·5 mg. of wool hydrolysate on starch columns eluted with 17% (v/v) aqueous 0·57N-HCl in *n*-butanol; 0·5 ml. fractions were collected (Stein & Moore, 1948). The valine peak coincided with that of methionine, and the methionine contribution to the combined peak was deducted to give the

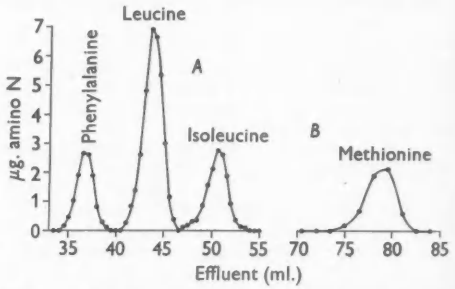

Fig. 1. Elution of amino acids from chromatogram starch I (Nin.) (see text). *A*, 3·5 mg. of wool hydrolysate for estimations of phenylalanine, leucine and isoleucine (0·5 ml. fractions); *B*, 10 mg. of wool hydrolysate for estimation of methionine only (1·5 ml. fractions).

valine content. The chromatogram is shown in Fig. 2 and referred to below as starch II. Tyrosine was also separated from 2 mg. of hydrolysate on a starch column eluted with 0·1 N-HCl, and 0·5 ml. fractions were collected (Moore & Stein, 1949). This chromatogram is referred to as starch III. Columns loaded with 3 mg. of wool hydrolysate and eluted with *n*-propanol–0·5 N-HCl (2:1, by vol.) were used for the separation of serine, glycine, arginine, lysine, histidine and cystine, 1 ml. fractions being collected (Moore & Stein, 1949). The arginine peak coincided with that of NH_3, but the latter was not estimated when the ^{64}Cu radiometric method of Blackburn & Robson (1953) was used to determine arginine. Although histidine was separated, it could not be accurately estimated in 3 mg. of hydrolysate. The chromatogram is shown in Fig. 3 and referred to later as starch IV. The amino acids separated on starch IV were also separated from 3 mg. of hydrolysate on a starch column eluted with *n*-butanol–*n*-propanol–0·1 N-HCl (1:2:1, by vol.) until 82 ml. had been collected, when the solvent was changed to *n*-propanol–0·5 N-HCl (2:1, by vol.) (Moore & Stein, 1949). 1 ml. fractions were collected. This chromatogram separates

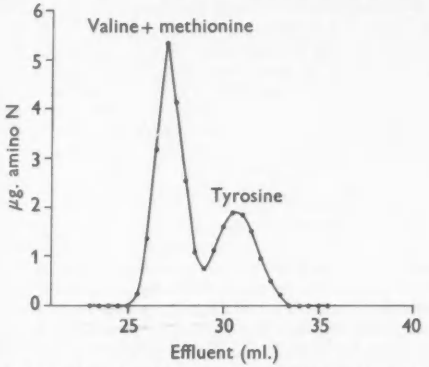

Fig. 2. Elution of valine, methionine and tyrosine from chromatogram starch II (Nin.) (see text). 2·5 mg. of wool hydrolysate; 0·5 ml. fractions.

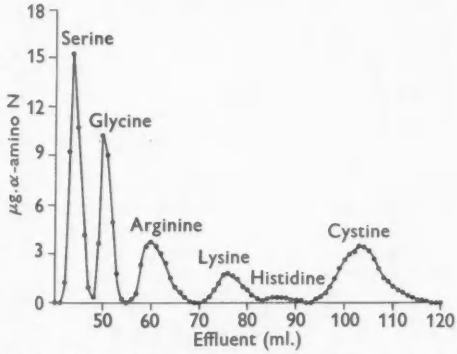

Fig. 3. Elution of serine, glycine, arginine, lysine, histidine and cystine from chromatogram starch IV (^{64}Cu) (see text). 3 mg. of wool hydrolysate; 1 ml. fractions.

arginine and NH_3, and is referred to as starch V. On chromatograms starch IV and starch V the alanine and glutamic acid peaks coincide, and threonine and aspartic acid are only partially separated. Experiments with various buffered solvent mixtures showed that good separations of proline, NH_3, threonine, alanine and glutamic acid could be achieved by eluting starch columns with *n*-butanol–*n*-propanol–citrate buffer (1:2:1, by vol.). The buffer (pH 3) was a mixture of 0·1 M disodium hydrogen citrate and 0·1 N-HCl (2:3, by vol.). 3 mg. of wool hydrolysate were applied and 1 ml. fractions collected. This chromatogram is shown in Fig. 4 and referred to as starch VI. No satisfactory means of completely separating aspartic acid from the other amino acids in wool hydrolysates on starch was found, and the aspartic acid contents were obtained by subtracting the estimated amount of threonine from the combined aspartic acid and threonine peaks obtained on starch IV and starch V.

Histidine, owing to its sparsity in wool hydrolysates, cannot be accurately determined by starch chromatography, as amounts of wool hydrolysate in excess of 3 mg. prevent its complete isolation from lysine and cystine. However, Dowex-50 columns 0·9 cm. in diameter and 15 cm. in length, prepared and eluted according to Moore & Stein (1951), enabled 19 mg. of wool hydrolysate to be fractionated with excellent resolution of the histidine and lysine peaks. This chromatogram is illustrated in Fig. 5 and referred to as Dowex-50.

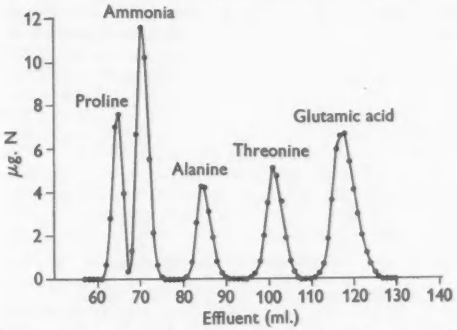

Fig. 4. Elution of proline, ammonia, alanine, threonine and glutamic acid from chromatogram starch VI (Nin.) (see text). 3 mg. of wool hydrolysate; 1 ml. fractions.

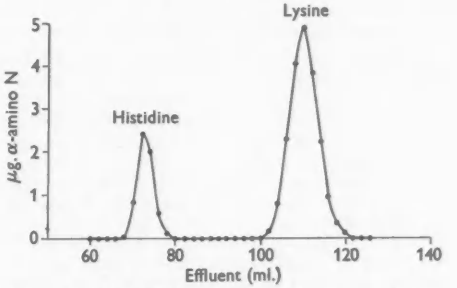

Fig. 5. Elution of histidine and lysine from chromatogram Dowex-50 (Nin.) (see text). 19 mg. of wool hydrolysate; 2 ml. fractions.

The recoveries of the amino acids from the seven different types of chromatogram, on separation from amino acid mixtures made to simulate a wool hydrolysate, were the same as those recorded by Moore & Stein, viz. $100 \pm 3\%$ for all except glutamic acid and aspartic acid, which were recovered in 93 and 94% yields, respectively. The recovery of aspartic acid was confirmed by starch chromatography with ^{14}C-labelled L-aspartic acid, when 94% of the total activity was recovered. The only exception to the above was the $100 \pm 3\%$ recovery of glutamic acid from starch VI, as there was no partial esterification of the acid in the buffered solvent.

Amino acid estimations were carried out in part by the photometric ninhydrin method of Moore & Stein (1948) and in part by the ^{64}Cu radiometric method of Blackburn & Robson (1953), as modified for use with starch chromatography by Corfield & Robson (1953). The latter method could not be used to estimate amino acids separated on starch I, as the solvent system prevented complete reaction between Cu^{2+} ions and amino acids and depressed the Cu^{2+} concentration over the copper phosphate suspension from 4×10^{-5} M to 2×10^{-6} M. Nor could it be used with fractions containing citrate, which forms complexes with Cu^{2+} ions. In all cases standard amino acid calibration curves were used, the data being obtained under reaction conditions identical with those for the column fractions. Calibration curves were repeated for each batch of ^{64}Cu because of changes in its specific activity, and also with different batches of ninhydrin reagent, which gave small variations in colour yields. All the amino acids used as standards were tested by filter-paper chromatography and elementary analysis, and shown to be free from amino acid impurities and from NH_3, sulphate and halide ions. The histidine and lysine standards were prepared from L-histidine monohydrochloride and L-lysine dihydrochloride respectively. In the presentation of the results, the chromatogram type has been stated with the abbreviated method of estimation in brackets, thus: starch IV (^{64}Cu); Dowex-50 (Nin.).

Other amino acid estimations

Tyrosine was estimated by the methods of Thomas (1944) and of Lugg (1937). Arginine was estimated by the method of Vickery (1940), glycine by the method of Alexander, Landwehr & Seligman (1945) and cystine by the method of Shinohara (1935). Serine and threonine were estimated by the method of Rees (1946).

Estimation of tryptophan

Tryptophan has proved to be the most difficult of all the amino acids in wool to estimate, as it is wholly or partially destroyed during hydrolysis. The methods of Lugg (1937), Brand & Kassell (1939), Sullivan & Hess (1944) and Spies & Chambers (1948, 1949) have been used in these laboratories, but the tryptophan determinations were extremely erratic.

No tryptophan is present in acid hydrolysates of wool, and the amounts present in alkaline hydrolysates vary with hydrolytic conditions. The only satisfactory method for tryptophan estimation was found to be that of Moore & Stein (1949) on chromatograms of the type starch III, where the tryptophan peak appears after tyrosine. 1 g. (dry wt.) samples of wool were sealed in Pyrex glass bulbs with 6·5 g. of recrystallized $Sr(OH)_2$, $8H_2O$ and 10·8 ml. of water, and heated in an oven at 100° for periods varying from 2 to 52 hr. Strontium was precipitated as $SrSO_4$ and removed by filtration, together with small amounts of SiO_2, and the filtrates were concentrated to 10 ml. Samples (0·19 ml.) of the hydrolysates were fractionated on starch III chromatograms. There was no interference with the tryptophan peak by slow-moving peptides in the partial hydrolysates, but when the 2 hr. hydrolysate was examined, a small peak, probably due to a peptide, appeared in front of the tryptophan. The relationship between the tryptophan found and the time of hydrolysis is shown in Fig. 6. The amount of tryptophan in wool was found by extrapolating the latter part of the curve back to zero time as shown.

Decomposition of the amino acids of wool during acid hydrolysis

Samples (approx. 0·2 g.) of glycine, DL-alanine, DL-valine, DL-leucine, DL-isoleucine, DL-phenylalanine, DL-serine, DL-proline, L-arginine, L-cystine, DL-threonine, L-tyrosine, L-histidine hydrochloride, L-lysine dihydrochloride, DL-glutamic acid, DL-aspartic acid and DL-methionine were refluxed separately for 24 hr. with 25 ml. of 5 N-HCl. The HCl was removed by vacuum distillation and the residues were dissolved in 50 ml. of water. The NH_3 contents of suitable samples of these solutions were determined with the apparatus of Markham (1942), using a suspension of $Mg(OH)_2$ to liberate NH_3. Other samples were made alkaline and aerated to remove any NH_3, brought to pH 5 with HCl, and their amino acid contents estimated by the photometric ninhydrin method (Moore & Stein, 1948).

Total N and ammonia

The total N contents of conditioned wool samples were determined (Chibnall, Rees & Williams, 1943). Digestion was for 16 hr. and twelve determinations were made. For the determination of amide N about 1 g. of conditioned wool was refluxed with 50 ml. of 2 N-HCl until the wool dissolved completely (usually 5 hr.). The solution was partly neutralized with 5 g. of KOH and diluted to 500 ml. 5 ml. of this solution were taken for estimation of NH_3 in the Markham apparatus as above. The NH_3 contents of 10 ml. samples of wool hydrolysates were likewise determined.

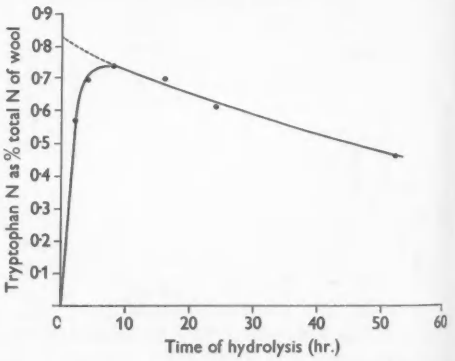

Fig. 6. Curve showing the amounts of tryptophan found after alkaline hydrolysis of wool for varying periods of time (see text).

Total S, ash and moisture

The total S of a conditioned wool sample was determined by the Benedict-Denis method (Barritt, 1934*a*). A weighed sample of conditioned wool was ashed to constant weight in a Pt crucible at 800°. To determine dry matter, a weighed sample of conditioned wool was heated to constant weight in an oven at 102°.

Wool sample and hydrolysis procedure

A quantity (6 lb.) of Australian Merino 64's quality virgin wool was 'tipped' by cutting off the top third of each lock of fibres to remove wool degraded by weathering. The root wool was extracted 6 times with the azeotropic mixture of benzene and methanol at 58·4°. After drying in air, each lock was washed separately in water at 45°, the bulk of the water removed in a centrifugal drier, and the wool air-dried for two days. Adhering vegetable matter was removed by combing the locks through a clean steel comb. The locks were drawn out by hand into slivers, gilled into one long sliver and chopped into pieces 1/32 in. in length on a special guillotine. After removing the last traces of grease by Soxhlet extraction with benzene–methanol, the wool was thoroughly stirred with distilled water (liquor to wool ratio, 10:1), and the water decanted.

The washing procedure was repeated 5 times and the wool finally separated on a large sintered glass filter. After drying in an oven at 40° to a water regain of about 8%, the sample was stored in stoppered bottles in the dark until required. Before use, a bottle was unstoppered and the wool conditioned for 1 week in a humidity room controlled at 15·5° and 65% relative humidity. Every time a sample was taken for analysis, a similar sample was removed for moisture-content determination.

With the exception of the hydrolysates prepared for tryptophan determinations, all hydrolysates were prepared by refluxing 1 g. (dry wt.) of wool for 24 hr. with 20 ml. of 5N-HCl, removing HCl *in vacuo* and making the residue up to 10 ml. with water.

RESULTS

The results of all the amino acid analyses are recorded in Table 1. Where amino acids were estimated by both the radiometric method and the ninhydrin method, the results show good agreement. The colour yields obtained for all the amino acids by the ninhydrin method were in accord with those of Moore & Stein (1948, 1949, 1951) except in the solvent mixture *n*-butanol–benzyl alcohol–water (1:1:0·280, by vol.) in which the colour yields of phenylalanine, leucine and isoleucine were low by 9, 9 and 6% respectively. Three different samples of DL-leucine, three of DL-isoleucine and two of DL-phenylalanine, and recrystallized specimens of these amino acids, were examined, but no variation in colour yield greater than 0·5% was observed. Purification of the solvents used made no difference to the colour yields.

The results of experiments carried out to determine the amounts of decomposition of the amino acids of wool during hydrolysis are shown in Table 2. Only serine, threonine, phenylalanine and arginine decomposed, and the arginine decomposition was insignificant. Threonine showed 8·3% decomposition when refluxed for 24 hr. with 5N-HCl, but only 3·5% loss could be accounted for as ammonia, on the basis of one mole of ammonia formed for one mole of threonine decomposed. This is contrary to the findings of Rees (1946), but agrees with recent work by Phillips (1954), who found that threonine decomposed to the extent of 7–9% with the liberation of 3% of its nitrogen as ammonia, under hydrolytic conditions slightly different from those we employed.

The ash content of the wool sample was 0·18%, its total nitrogen content 16·35%, its amide nitrogen content 1·10% and the average ammonia-nitrogen content of its acid hydrolysates 1·40%.

DISCUSSION

The estimations of serine and threonine by the method of Rees (1946), of tyrosine by the methods of Lugg (1937) and Thomas (1944), and of arginine by the method of Vickery (1940) were in good agreement with the chromatographic estimations, but glycine estimated according to Alexander *et al.* (1945) gave a value 15% lower than the chromatographic figure. In this case the latter is considered to be nearer the true value, since complete separation of glycine from the hydrolysate removes possible sources of interference with its estimation. The chromatographic estimation of cystine was 20% lower than the amount found by the method of Shinohara (1935), and if the latter estimate is correct the 3·38% sulphur content of the wool sample can be wholly accounted for as methionine sulphur and cystine sulphur. This is in accord with similar analyses made in these laboratories and elsewhere (Rimington, 1929; Barritt, 1934*b*; Bailey, 1937; Cuthbertson & Phillips, 1945). On the other hand, Stein & Moore (1949) found that the cystine contents of hydrolysates of β-lactoglobulin and bovine serum albumin were 10% lower than cystine determined by other methods, and the figure for cystine in wool hydrolysates found by Simmonds (1954) using ion-exchange resin chromatography (Moore & Stein, 1951) was 38% lower than the amount he found by Shinohara's method. Stein & Moore (1949) stated that their cystine results may be low because of the differences in times of hydrolysis of the two samples. However, in our experience with wool hydrolysates, the Shinohara method gives the same cystine value for times of hydrolysis from 4 to 24 hr. Another method for cystine estimation as, for example, an isotope-dilution method, would furnish independent analyses and help to resolve the problem. Such a method is being investigated in our laboratories at

Table 1. *Amino acid analyses of wool hydrolysates*

Amino acid	Hydrolysate	Method of separation and estimation	No. of estimations	Amino acid N (as % total N)	Average amino acid N (as % total N)	Amino acid/100 g. wool
Alanine	A	Starch VI (Nin.)	3	4·05±0·04	4·12	4·3
	B	Starch VI (Nin.)	3	4·20±0·04		
Arginine	A	Starch V (Nin.)	2	19·4±0·4	19·1	9·8
	B	Starch V (Nin.)	2	18·8±0·2		
	C	Starch IV (^{64}Cu)	1	19·2		
	D	Starch IV (^{64}Cu)	1	19·6		
	F	Vickery (1940)	2	21·2±0·2	20·6	10·4
	G	Vickery (1940)	2	20·1±0·1		
Aspartic acid	A	Starch V (Nin.)	2	4·15±0·07	4·38	6·8
	A	Starch V (^{64}Cu)	1	4·51		
	B	Starch V (Nin.)	2	4·54±0·19		
	B	Starch V (^{64}Cu)	1	4·35		
Cystine	C	Starch IV (^{64}Cu)	1	7·26	7·30	10·3
	E	Starch IV (^{64}Cu)	1	6·73		
	D	Starch IV (^{64}Cu)	1	7·50		
	E	Starch IV (Nin.)	1	7·67		
	A	Starch IV (Nin.)	2	7·52±0·02		
	A	Starch V (Nin.)	1	6·83		
	A, B, H, I, J, K	Shinohara (1935)	6	8·73±0·04	8·73	12·3
Glutamic acid	A	Starch VI (Nin.)	3	8·53±0·17	8·48	14·5
	B	Starch VI (Nin.)	3	8·44±0·16		
Glycine	C	Starch IV (^{64}Cu)	1	6·14	6·29	5·5
	D	Starch IV (^{64}Cu)	1	6·61		
	A	Starch IV (Nin.)	2	6·55±0·39		
	B	Starch IV (Nin.)	1	6·35		
	A	Starch V (Nin.)	2	5·97±0·12		
	B	Starch V (Nin.)	2	6·28±0·15		
	D	Alexander *et al.* (1945)	6	5·48±0·05	5·45	4·8
	E	Alexander *et al.* (1945)	5	5·43±0·08		
	C	Alexander *et al.* (1945)	6	5·45±0·10		
Histidine	A	Dowex-50 (Nin.)	1	1·87	1·91	1·2
	B	Dowex-50 (Nin.)	3	1·93±0·01		
Isoleucine	E	Starch I (Nin.)	1	2·50	2·44	3·7
	D	Starch I (Nin.)	3	2·40±0·06		
	C	Starch I (Nin.)	2	2·48±0·06		
Leucine	E	Starch I (Nin.)	1	5·90	5·85	8·9
	D	Starch I (Nin.)	3	5·86±0·06		
	C	Starch I (Nin.)	2	5·80±0·16		
Lysine	B	Starch V (Nin.)	2	3·74±0·04	3·92	3·3
	C	Starch IV (^{64}Cu)	1	4·32		
	D	Starch IV (^{64}Cu)	1	3·67		
	E	Starch IV (^{64}Cu)	1	3·91		
	E	Starch IV (Nin.)	1	3·86		
	A	Starch IV (Nin.)	2	3·70±0·06		
	B	Starch IV (Nin.)	1	3·77		
	A	Starch V (Nin.)	1	4·26		
	A	Dowex-50 (Nin.)	1	4·05		
	B	Dowex-50 (Nin.)	1	4·21		
Methionine	D	Starch I (Nin.)	1	0·314	0·32	0·56
	A	Starch I (Nin.)	3	0·320±0·008		
	B	Starch I (Nin.)	2	0·318±0·018		
Phenylalanine	E	Starch I (Nin.)	1	2·20	2·07	4·0
	D	Starch I (Nin.)	3	2·02±0·04		
	C	Starch I (Nin.)	2	2·11±0·02		
Proline	A	Starch VI (Nin.)	3	4·96±0·03	5·05	6·8
	B	Starch VI (Nin.)	1	5·04		
	A	Starch V (^{64}Cu)	1	5·18		
	B	Starch V (^{64}Cu)	1	5·35		
	A	Starch V (Nin.)	2	5·03±0·15		
	B	Starch V (Nin.)	2	4·98±0·15		

Table 1 (*cont.*)

Amino acid	Hydrolysate	Method of separation and estimation	No. of estimations	Amino acid N (as % total N)	Average amino acid N (as % total N)	Amino acid/100 g. wool
Serine	L	Rees (1946)	1	8·41	8·07	9·9
	M	Rees (1946)	5	8·00±0·12		
	C	Starch IV (^{64}Cu)	1	8·51	7·87	9·7
	D	Starch IV (^{64}Cu)	1	7·97		
	A	Starch IV (Nin.)	2	8·14±0·06		
	B	Starch IV (Nin.)	1	7·77		
	A	Starch V (Nin.)	2	7·51±0·06		
	B	Starch V (Nin.)	2	7·66±0·08		
Threonine	A	Starch VI (Nin.)	3	4·74±0·16	4·71	6·5
	B	Starch VI (Nin.)	3	4·69±0·04		
	L	Rees (1946)	2	4·55±0·05	4·56	6·3
	M	Rees (1946)	5	4·56±0·02		
Tryptophan	See text	Starch III (Nin.)	See text	—	0·82	0·94
Tyrosine	A	Starch III (^{64}Cu)	1	2·57	2·62	5·5
	B	Starch III (^{64}Cu)	1	2·66		
	A	Starch III (Nin.)	2	2·64±0·01		
	B	Starch III (Nin.)	2	2·62±0·09		
	N	Lugg (1937)	8	2·83±0·03	2·79	5·9
	O	Lugg (1937)	8	2·75±0·03		
	P	Thomas (1944)	3	2·63±0·01	2·64	5·6
	Q	Thomas (1944)	2	2·65±0·01		
	R	Thomas (1944)	2	2·63±0·03		
Valine	A	Starch II (^{64}Cu)	1	4·24	4·16	5·7
	B	Starch II (^{64}Cu)	1	4·21		
	E	Starch II (Nin.)	3	4·20±0·04		
	D	Starch II (Nin.)	3	4·08±0·06		
	C	Starch II (Nin.)	2	4·15±0·02		

Table 2. *Losses of amino acids during acid hydrolysis*

For further details see text.

Amino acid	Recovery (%)	Ammonia formed (N as % total N)
Serine	90·8	9·1
Threonine	91·7	3·5
Phenylalanine	97·6	0·5
Arginine	100	0·3

present. Although the chromatographic estimation of 10·3 g. cystine/100 g. wool cannot account for the total sulphur in wool we prefer to record it as being nearer the true figure than the Shinohara value.

The results of the present amino acid analysis of wool are given in Table 3, together with the analyses by Simmonds (1954) and by Graham *et al.* (1949). The figures for serine, threonine and phenylalanine shown in Table 3 have been corrected by the factors 100/90·8, 100/91·7 and 100/97·6, respectively, in accordance with the losses of these amino acids in recovery experiments (Table 2). Whilst the correction factors are probably too low, because there is evidence, at least for serine, that amino acid residues decompose more rapidly when combined than in the free state (e.g. Corfield & Robson, 1953), the exact amounts of decomposition cannot be determined.

There is an excess of ammonia in wool hydrolysates over that due to amide hydrolysis, but this cannot confidently be used as a measure of the decomposition of threonine, serine and phenylalanine as there is some uncertainty in the amide ammonia estimation. It is certain, however, that the whole of the excess ammonia cannot be accounted for if the rates of decomposition of serine, threonine, phenylalanine and arginine are no greater during the hydrolysis of wool than under the conditions of the experiments in Table 2.

The agreement between our amino acid analyses and those of Simmonds (1954) is not so close as might have been expected in view of the fact that they were both obtained from samples of Australian 64's quality wool. In particular, his estimations of alanine, histidine, isoleucine, leucine, lysine, phenylalanine and valine are 20 % or more lower than our figures. The nitrogen balances of the two analyses are both close to 100 %, but that of Simmonds includes the nitrogen from two unidentified peaks he found on his chromatograms, one of which is probably due to hydroxylysine. This amino acid has been found previously in wool hydrolysates by Van Slyke, Hiller & McFadyen (1941) and its presence confirmed by Middlebrook (1949), but it comprises only 0·18 % of the wool. No unidentified peaks were found on starch chromatograms, but it is

Table 3. *Amino acid analyses of wool by various authors*

	N as % total N of wool		
Amino acid	Simmonds (1954)	Present work	Graham, Waitkoff & Hier (1949)
Alanine	3·51	4·12	—
Amide N	7·46*	6·73	—
Arginine	20·3	19·1	21·1
Aspartic acid	4·24	4·38	4·7
Cystine	7·93 (4·95)	7·30	9·9
Glutamic acid	8·58	8·48	9·2
Glycine	5·80	6·29	—
Histidine	1·46	1·91	1·8
Isoleucine	1·97	2·44	3·0
Leucine	4·90	5·85	5·3
Lysine	3·25	3·92	3·9
Methionine	0·39	0·32	0·4
Phenylalanine	1·75	2·12†	2·1
Proline	5·33	5·05	6·1
Serine	7·25	8·66†	—
Threonine	4·61	5·12†	4·8
Tryptophan	1·73	0·82	—
Tyrosine	2·97	2·62	2·7
Valine	3·57	4·16	4·2
Unknown (1)	1·18	—	—
Unknown (2)	0·71	—	—
Total	98·89	99·39	79·2

* Uncorrected for decomposition of serine and threonine during hydrolysis.

† Corrected for loss during hydrolysis.

possible that an unknown, present in small amount, might have coincided with a known amino acid peak and thereby escaped detection. The total N and total S contents of the two wool samples are also different.

On the whole our analyses agree well with those of Graham *et al.*, the agreement for five amino acids being within ± 5 % and for ten within ± 10 %, with larger discrepancies in the estimations of proline, isoleucine, cystine and methionine. This last only occurs to the extent of 0·5–0·7 % in wool and is consequently difficult to determine accurately, and the large discrepancies between cystine estimations obtained by different methods have already been mentioned.

As Simmonds has pointed out, wool is almost certainly not a homogeneous protein, and differences in the amino acid composition of different wool samples are to be expected. This contention is supported by the analytical data shown in Table 3.

SUMMARY

1. The amino acid composition of a sample of Australian Merino 64's quality wool has been determined.

2. The results obtained have been compared with recent amino acid analyses of wool.

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Purification and Properties of Some Enzymes in Human Seminal Plasma

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Among the enzymes in human seminal plasma only the acid phosphatase has so far been extensively studied, while the proteolytic enzymes have been the subject of only few investigations. Huggins & Neal (1942) examined in some detail the fibrinolytic properties of semen and prostatic secretion, and also claimed that 'trypsin' is present in small amounts. Oettlé (1950) confirmed the presence of a fibrinolytic enzyme, for which he proposes the name 'seminin' by analogy with the plasmin of blood plasma. He also found in semen a heat-stable inhibitor of fibrinolysis. Treatment with streptokinase gave no evidence of the presence of an enzyme precursor, and furthermore he found no kinase similar to the fibrinokinase of other tissues. On the other hand, von Kaulla & Shettles (1953) reported the presence both of an enzyme precursor (similar to plasminogen) and a kinase.

Lundquist (1952) described a casein-hydrolysing enzyme which is probably identical with the 'trypsin' of Huggins & Neal. The formation of large amounts of free amino acids from a fibrin-like material produced in the vesicular glands was demonstrated. No physiological function has yet been attributed to the amino acids in semen. Evidence that at least two enzymes are concerned in this proteolytic process has been presented (Lundquist & Buus, 1952; Lundquist, 1953). Pepsinogen, or an enzyme precursor closely resembling pepsinogen, was found in human semen by Lundquist & Seedorff (1952).

In the present work we have aimed at the separation and characterization by means of low-molecular synthetic substrates of two proteolytic enzymes in semen, a proteinase and an aminopeptidase. During our work with these enzymes we have also obtained the prostate phosphatase in a state of high purity.

EXPERIMENTAL

Materials

The enzyme preparations used as starting material in all attempts at purification and separation of the proteolytic enzymes of human seminal plasma were made from batches of semen collected at a fertility clinic. Batches (50–70 ml.) of semen were received each week and centrifuged to remove cells. The plasma was mixed with 0·1 vol. 3M acetate buffer, pH 5. Saturated $(NH_4)_2SO_4$ soln. was added to make a final concn. corresponding to 40% saturation ($\frac{2}{3}$ vol.). After 1 or 2 hr. in the refrigerator the mixture was centrifuged. Saturated $(NH_4)_2SO_4$ was again added to the supernatant to a final saturation of 70%. The precipitate was collected by centrifuging and was sucked nearly dry on a Büchner funnel placed in the refrigerator. The material was dissolved in a small amount of water and dialysed in cellophan tubes against distilled water at 5° for 24 hr. The precipitate formed was removed by centrifuging. The volume of this enzyme preparation was generally about half that of the starting material. For storage over prolonged periods the enzyme solution was kept frozen at −20°. Some loss in proteinase activity occurred by this treatment, while peptidase and phosphatase activities remained stable.

Synthetic substrates. The following substrates were synthesized (for methods see references in parentheses): glycylglycine (Schott, Larkin, Rockland & Dunn, 1947); diglycylglycine from chloroacetylglycylglycine (Fischer, 1903, 1906); glycyl-L-leucine (Abderhalden & Weber, 1910); benzoylglycine amide (Franzen, 1909); L-leucine amide (Smith & Slonim, 1948); benzoyl-L-arginine ethyl ester and benzoyl-L-arginine amide (Bergman, Fruton & Pollok, 1939) (it was found that the yield in the last case could be nearly doubled by transforming directly, without isolation, the benzoylarginine ethyl ester into the amide); chloroacetyl-L-tyrosine (Fischer, 1904); L-tyrosine ethyl ester (Fischer, 1901).

Acetyl-L-tyrosine ethyl ester was prepared from the tyrosine ester by boiling with the equivalent amount of acetic anhydride in an excess of dry benzene. In the preparation of *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosine (Bergman, Zervas, Salzmann & Schleich, 1934), it was found advantageous to use equivalent amounts of L-tyrosine and benzyloxycarbonyl-L-glutamic anhydride in ethyl acetate for the coupling; the yield was 71%. Benzyloxycarbonylglycyl-L-phenylalanine was made as described for benzyloxycarbonyl-L-leucylglycylglycine (Schott *et al.* 1947). DL-Alanylglycine and L-leucylglycine were commercial products (Light and Co Ltd.). DL-Alanylglycylglycine was a gift from Professor Linderstrøm-Lang, the Carlsberg Laboratories, Copenhagen. The ethyl esters of L-cysteine, *N*-phenylglycine and acetyl-DL-alanine were prepared in the usual way by dissolving in ethanol saturated with HCl and boiling for 30 min. Phenylpropionic acid (hydrocinnamic acid) was recrystallized twice for use in inhibition experiments. Melting points and nitrogen content were, for all the substances used, in agreement with the data in the literature or close to the theoretical values respectively.

Analytical methods

Measurement of the hydrolysis of casein. The procedure used has been described by Lundquist (1952).

Measurement of hydrolysis of synthetic substances. Estimations of peptidase activity were performed by titration with ethanolic HCl in acetone according to Linderstrøm-Lang (1927) and Linderstrøm-Lang & Holter (1931). The following procedure was used. 1 ml. enzyme solution is mixed with 1 ml. 0·1 M substrate solution containing 0·02 M phosphate buffer (generally a pH value of 7·7 was used). Duplicate determination of the initial titration value is performed immediately on samples taken out with a 0·2 ml. Carlsberg constriction pipette. The mixture is incubated at 37° in tubes closed with rubber stoppers. At convenient times 0·2 ml. samples are removed and the enzymic process is stopped by addition of ethanolic HCl from a calibrated Carlsberg pipette. Titrations are made from a Rehberg microburette with 0·1 N-HCl in 90% ethanol after addition of 2 ml. acetone–indicator mixture. Control experiments with enzyme without substrate and substrate without enzyme were performed frequently. These blank values were in nearly all cases insignificant. In most cases the reaction was first order and the results are expressed as the first-order reaction constant (average of three samples). Titrations with ethanolic KOH according to Grassmann & Heyde (1929) were performed in a few experiments with acetyltyrosine ethyl ester. The titrations were, however, not very satisfactory, as a high concentration of buffer had to be used, resulting in large blank values and an ill-defined endpoint because of precipitation in the ethanolic medium. We have therefore preferred a manometric method, described below, which was found to be definitely superior.

Manometric technique. The hydrolysis of the ester bond of acetyltyrosine ethyl ester and benzoylarginine ethyl ester is conveniently followed manometrically. A similar method for the determination of chymotrypsin using phenylalanine ethyl ester as substrate has recently been described by Parks & Plaut (1953). The concentrations used in our experiments were: buffer, 0·04 M-$NaHCO_3$; gas phase, 95% N_2 + 5% CO_2; substrate concentration, 0·05 M in the case of benzoylarginine ethyl ester and other soluble amino acid esters (L-leucine, L-cysteine ethyl ester and *N*-phenylglycine ethyl ester). The total volume of liquid in the Warburg vessels was 3 ml. in all cases. The substrate used for measuring chymotrypsin-like activity was acetyl-L-tyrosine ethyl ester, which is only slightly soluble in water. Kaufman, Neurath & Schwert (1949), who have established the value of this and similar esters as substrates for chymotrypsin, made their measurements in 30% methanol. In this medium the apparent enzymic activity of chymotrypsin towards benzoyltyrosine ethyl ester is approximately 10 times less than without addition of methanol. A number of our experiments were performed with the manometric technique using an overall concentration of methanol of 30% (v/v). 0·5 ml. of 0·3 M substrate solution in methanol was placed in the side arm of the Warburg vessel, the main compartment of which contained sufficient methanol to bring its final concentration to 30% (v/v). As the solubility of CO_2 in aqueous methanol is different from that in water, it was necessary to measure the flask constants in separate experiments, where known amounts of acid were added from the side arm to Warburg flasks containing $NaHCO_3$ in 30% methanol. The sensitivity of the method was considerably improved by using water as the solvent and having the substrate present as a fine suspension. 0·5 ml. of an aqueous suspension of the finely powdered substance (containing approximately 0·25 mole/l.) is measured into the side arm and the experiment performed in the usual way. The velocity of the reaction is increased at least 10 times on changing from the 30% methanol medium to water. Moreover, the enzymes are more stable under these conditions and the reaction proceeds at a constant rate during the whole experiment. The reaction is followed throughout a period varying from 15 to 150 min. In all experiments with seminal enzyme preparations we have found the hydrolysis to proceed at a constant rate, but in control experiments with pure (crystalline) trypsin ('Novo') and chymotrypsin (a gift from Dr Astrup, The Biological Institute of the Carlsberg Foundation) a rapid decrease in reaction velocity was observed. This is probably due to surface denaturation in the very dilute enzyme solution. Addition of gelatin had considerable stabilizing effect, but is of course, undesirable.

Measurement of fibrinolysis. For determination of fibrinolytic activity we have employed a slight modification of the fibrin-plate method of Astrup & Permin (1947), and Permin (1950). Ox fibrinogen, purified by two precipitations with $(NH_4)_2SO_4$ and in some cases further purified by precipitation with an equal volume of 2 M phosphate buffer (Jacques, 1943), is coagulated in a 2·5 mm. thick layer in Petri dishes, chosen to be nearly flat. The concentration of the fibrinogen solution is 0·3% and the coagulation is brought about by addition of purified thrombin (a preparation from 'Leo' Ltd., Copenhagen). A number of holes (usually 5) are cut in the coagulum by means of a cork borer, 5 mm. in diameter, and 50 µl. of enzyme are placed in each hole. The dishes are incubated at 38° for 24 hr. and the areas of the lysed zones are measured. The dishes are photographed by placing them on photographic paper (for contact printing) and exposing them to light from an ordinary desk lamp placed vertically over the dish. This could be performed inside the incubator without unduly moving the dishes. The lysed area was measured as the difference between the products of the longest and shortest diameters (when the holes were not perfectly circular) after and before incubation. This area was found within certain limits to be nearly proportional to the enzyme concentration. An enzyme preparation contains 1 unit/ml. if the assay performed as described results in an increase in the diameter-product of 1 $cm.^2$ in 24 hr. at 38°.

As bovine fibrin contains a precursor (plasminogen) of the fibrinolytic enzyme plasmin, this kind of experiment does not decide whether a fibrinolytic enzyme is present in a sample causing lysis on the plates, or whether we have an activator which catalyses the formation of plasmin from plasminogen. In order to differentiate between these two possibilities, some experiments were performed using the technique of Lassen (1952), who found that heating of the fibrin plates to 90° for 30 min. destroys the plasminogen present in the fibrin, without seriously impeding the availability of the fibrin as a substrate for proteolytic enzymes such as trypsin.

Zone electrophoresis

Several modifications of this technique employing either filter paper or glass beads and potato starch have been tried. In the experiments with filter paper the procedure of Kunkel & Tiselius (1952) was used. Generally a few layers of Whatman no. 1 paper (40 cm. long, 8 cm. wide) were placed on top of one another, one piece was used for staining with bromophenol blue, while the others were cut in strips 1 cm. wide, and eluted for enzyme determinations. The technique

used for glass beads and starch electrophoresis was a modification of that of Kunkel & Slater (1952). A Perspex apparatus was constructed consisting of a trough of internal dimensions $40 \times 10 \times 1{\cdot}5$ cm. The trough is connected through vertical slits with the solutions in the electrode vessels (cf. Fig. 1). A tightly fitting Perspex plate, greased with a little vaseline, is placed on top of the apparatus when in use.

The most effective material for supporting the buffer used in the electrophoresis was found to be potato starch, which gives rise to only very small electro-osmotic flow and hardly any 'tailing'. 500 g. of potato starch (best available commercial grade) are suspended in 5 l. of distilled water and the water is decanted after most of the material has settled. This treatment is repeated 4–5 times and followed by 2–3 similar treatments with the buffer to be used in the experiment. The starch is now suspended in sufficient buffer to make a thick paste, which is poured into the plastic trough. Before the starch is added, a Perspex plate 3 mm. thick was fixed in a vertical position about 10 cm. from the cathode end of the trough and perpendicular to the direction of the current. After settling for 10–20 min. the excess of buffer is removed from the starch mass by means of filter paper, until a hard uniform block is formed. An air space of about 2 mm. should remain between the surface of the starch and the lid. The plate is now removed cautiously, leaving a clean, dry groove in the material. A thick suspension is prepared, consisting of starch, purified as described and sucked nearly dry, and the protein solution to be examined. This suspension is poured into the slit. The whole apparatus is placed in the refrigerator (at 5°) and after equilibration for 2 hr. the electrodes are connected to the 220 v d.c. mains. The potential gradient through the starch block was usually 3 v/cm. The composition of the buffer solution has been varied, but in the most successful experiments it was as follows: aminotrishydroxymethylmethane, 0·05 M; NaCl, 0·03 M; HCl added to pH 8·1. The enzyme solutions were freeze-dried after thorough dialysis against water, and *ca.* 200 mg. of the dry substance were dissolved in about 2 ml. of the same buffer before mixing with dry starch. Duration of the electrophoresis, 20 hr.

For analysis, the starch block is cut into segments exactly 1 cm. wide. The cutting and transference to numbered glass tubes is performed by means of two Perspex plates 10 cm. wide with a thin straight edge. One plate is used for cutting off the segments while the other is used for the transfer. This procedure can be made very accurate, and 5 mm. segments can easily be cut, if better resolution is desired. The segments are eluted with a suitable buffer or salt solution. Generally 10 ml. of solvent were used for 1 cm. segments. Centrifuging is not necessary, as the starch settles to the bottom of the tube quite rapidly after stirring.

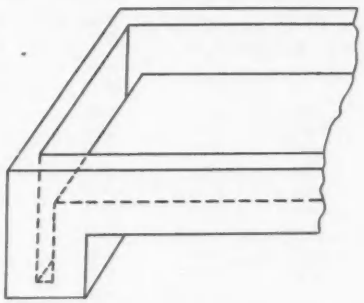

Fig. 1. Detail of Perspex trough for zone electrophoresis. The vertical slit, filled with cotton and buffer solution, connects the trough with the electrode vessel (not shown). Dimensions are given in text.

Fractionation by adsorption on tricalcium phosphate gel

The procedure finally adopted in our fractionation experiments is based on the work of Swingle & Tiselius (1951). Instead of the chromatographic technique we have, however, used adsorption followed by successive elution with salt–buffer solutions, of increasing ionic strength. The $Ca_3(PO_4)_2$ gel was prepared according to MacIntire, Palmer & Marshall (1945) and used after ageing for 3 months. 80–100 ml. of enzyme solution were mixed thoroughly with an equal volume of $Ca_3(PO_4)_2$ suspension (containing 39 mg. dry matter/ml.). After centrifuging, only a small amount of protein (10–15%) and no measurable enzyme activity is present in the supernatant. The precipitate is extracted twice with 0·01 M phosphate buffer, 3 times successively with each of: 0·02 M, 0·033 M, and 0·067 M phosphate buffer, and finally 3 times with 0·067 M phosphate containing 2% NaCl. The pH of all solutions was 7·8 and 25 ml. were used each time. For extraction the precipitate is stirred vigorously with a mechanical stirrer in a plastic tube until the mixture appears homogeneous. The mixture is centrifuged for 10 min. at 3000 rev./min. During the whole procedure the temperature was kept at 5–10°.

Other methods

Protein determinations were made with the micro-Kjeldahl method or by measuring the optical density at 280 mμ. In the latter case the specific extinction of the protein mixture (optical density/mg. N) was always determined. Phosphatase measurements were made with phenyl phosphate as substrate at pH 5·5 in citrate buffer. Incubation time, 15 min. temp., 37°. The phenol liberated was measured with phenol reagent (Folin & Ciocalteu, 1927). One unit (Gutman & Gutman, 1941) is defined as the amount of enzyme which liberates 1 mg. of phenol/hr. under these conditions.

RESULTS

In order to characterize the proteolytic enzymes in semen, a number of synthetic peptides, amides and esters were employed. Table 1 shows the results of experiments with some of the substrates used in this study. It is clear that carboxypeptidase is entirely absent, whereas peptides with a free amino group are split. The substrate specifically attacked by pepsin is not appreciably hydrolysed, though it is known (Lundquist & Seedorff, 1952) that pepsinogen does in fact occur in human semen. The reason for this absence of hydrolysis of benzyloxycarbonylglutamyltyrosine is that pepsinogen is nearly quantitatively removed during the preparation of the enzyme. Moreover, the substrate used is not a very sensitive indicator for pepsin.

The activity towards the substrates for trypsin and chymotrypsin is weak or absent in the case of the amides but considerable with the esters, which are much more sensitive to trypsin and chymotrypsin respectively than the amides. Schwert, Neurath, Kaufman & Snoke (1948) state that benzoyl-L-arginine ethyl ester is hydrolysed about 60 times as fast as the corresponding amide, and Kaufman *et al.* (1949) find chymotrypsin to be more than 1000 times as active towards ester substrates than towards amides. The specificity of the substrates as indicators of hydrolysis by trypsin and chymotrypsin is, however, far from absolute.

Separation of individual enzymes. Separation by means of salt fractionation and fractionation with organic solvents at low temperatures was tried but without appreciable success. Some purification of the peptidase was achieved with acetone fractionation, but there was no clear-cut separation. The use of tricalcium phosphate gel as adsorbent, followed by elution with buffer solutions of increasing ionic strength did, however, result in considerable purification and separation of the enzymic activity towards the small peptides on the one hand, and the activity in tests with casein and acetyltyrosine ester on the other. Furthermore,

Table 1. *The activity of crude seminal enzyme towards selected synthetic substances*

Substrate	Preferentially hydrolysed by	Duration of expt.	First-order reaction const. ($10^3\,k$)
Benzyloxycarbonyl-L-glutamyl-L-tyrosine	Pepsin	18 hr.	No hydrolysis
Chloroacetyl-L-tyrosine	Carboxypeptidase	18 hr.	No hydrolysis
Benzyloxycarbonyl-glycyl-L-phenylalanine	Carboxypeptidase	18 hr.	No hydrolysis
Benzoyl-L-arginine amide	Trypsin	18 hr.	No hydrolysis
Benzoyl-L-arginine ethyl ester	Trypsin	30 min.	16 μmole/hr.†
Glycylglycylglycine	Peptidases	30 min.	8·3
DL-Alanylglycylglycine*	Peptidases	20 min.	28
DL-Alanylglycine*	Peptidases	60 min.	5·8
L-Leucylglycine	Peptidases	120 min.	0·66
Glycyl-L-leucine	Peptidases	150 min.	0·55
Glycylglycine	Peptidases	150 min.	0·94
L-Leucine amide	Leucylaminopeptidase	120 min.	2·5
Acetyl-L-tyrosine amide	Chymotrypsin	17 hr.	0·035
Acetyl-L-tyrosine ethyl ester	Chymotrypsin	30 min.	13 μmole/hr.†
Benzoylglycine amide	'Endopeptidase'	18 hr.	No hydrolysis

* The reaction constants for the DL-alanylpeptides are calculated on the basis of the L-forms, as it was found that the reaction stopped completely at 50% hydrolysis.
† Manometric experiments. The reactions follow a zero-order reaction scheme.

Table 2. *Fractionation of enzyme by tricalcium phosphate adsorption*

Fraction no.	Eluent	Vol. (ml.)	Total N (mg. N/ml.)	Phosphatase (units/ml.)	Casein hydrolysis (units/ml.)	Leucine amide hydrolysis ($10^3\,k$)	'Fibrinase' (units/ml.)
1	Starting material	90	1·66	4830	3·69	2·08	8·6
2	Supernatant after adsorption	140	0·219	0	0	0	0
3	0·01 M phosphate	30	0·198	0	0	0	0
4	0·01 M phosphate	16·5	0·218	0	0	0	0
5	0·02 M phosphate	25	0·239	435	0	1·61	0
6	0·02 M phosphate	26	0·285	1650	0·18	2·86	0
7	0·02 M phosphate	26·5	0·285	2340	0·19	2·36	0
8	0·033 M phosphate	26·5	0·330	3190	0·19	1·75	0
9	0·033 M phosphate	27	0·322	1995	0·21	1·35	0
10	0·033 M phosphate	29·5	0·217	1460	0·20	0·66	0
11	0·067 M phosphate	25	0·333	1165	0·52	0·52	1·8
12	0·067 M phosphate	28	0·281	1215	0·48	0·19	3·0
13	0·067 M phosphate	25	0·306	965	0·67	0·22	4·1
14	0·067 M phosphate + 2% NaCl	26	0·331	0	0·66	0·10	7·6
15	0·067 M phosphate + 2% NaCl	26	0·280	0	0·38	0·09	8·0
16	0·067 M phosphate + 2% NaCl	23	0·212	0	0·17	0·06	6·3
Recovery (% of starting material)			87	89	31	107	101
Maximum purification				3·3	None	5	5·7

a fibrinolytic enzyme, or perhaps rather an activator of plasminogen (see later), was separated from the bulk of the material, and the acid prostate phosphatase was purified quite effectively.

Table 2 shows an experiment of this kind. A number of similar experiments were done to compare the activities towards different substrates. About 20% of the nitrogen remains in the fluid after adsorption; this fluid is apparently devoid of enzymic activity. About 10% of the nitrogen adheres to the tricalcium phosphate after the final elution. The acid phosphatase was usually recovered nearly quantitatively, and the same was the case with the peptidase and the plasminogen activator; only 30–50% of the proteinase which hydrolyses casein and acetyltyrosine ethyl ester was recovered, and, moreover, the purification on a nitrogen basis of the proteinase was in most cases insignificant. Experiments of this kind, where the fractions were assayed both against casein and acetyltyrosine ethyl ester, showed complete parallelism between the two activities. In other experiments the fractions were tested against glycylglycine, glycylleucine, leucylglycine, triglycine and alanylglycine, and these activities ran parallel in all fractions, as is apparent from Fig. 2.

For further purification of peptidase, proteinase, phosphatase and fibrinase, two or three of the fractions with highest enzyme activity were combined, precipitated with ammonium sulphate, dialysed and freeze-dried. The material thus obtained was subjected to filter-paper electrophoresis. In this way the four activities could be readily separated, but very marked losses occurred, especially of the proteinase, which appears to be a rather unstable enzyme. It was, however, found later in this investigation that equally good results might be obtained by subjecting the crude enzyme to electrophoresis on a starch medium without previous tricalcium phosphate fractionation. The results of an experiment of this kind are shown in Fig. 3. Three well-defined peaks were found. The first coincides with the peak of activity towards casein and acetyltyrosine ethyl ester, the second coincides exactly with the curve for phosphatase activity, but in the case of the last peak no enzymic counterpart was found. The activity

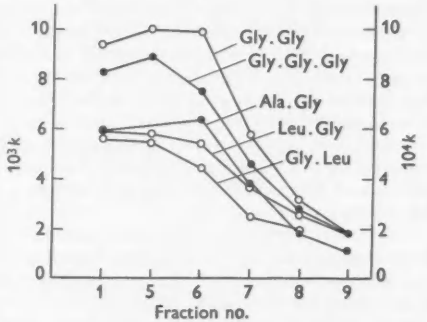

Fig. 2. Activity of fractions from a $Ca_3(PO_4)_2$ adsorption towards a number of peptides. Technique as described in Experimental section. The results are plotted as first-order reaction constants. Circles correspond to the right ordinate axis, dots to the left axis. Fraction numbers have the same significance as in Table 2. The value for alanylglycine in fraction 1 is based on only one pair of samples and it may be somewhat too low.

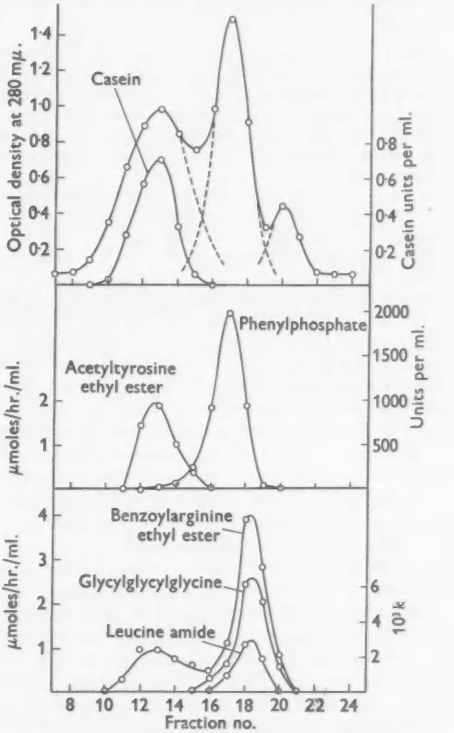

Fig. 3. Electrophoresis experiment with crude seminal enzyme. Supporting material: potato starch. Buffer: 0·05 M aminotrishydroxymethylmethane (pH 8·1) + 0·03 M-NaCl. Duration of experiment: 20 hr., temp. 7°. Protein was applied at the position corresponding to fraction 11. The upper curve corresponds to the left ordinate axis and gives the optical density at 280 mμ. All other curves depict the activities of the various fractions towards the substrates indicated on the curves. Casein, phenyl phosphate, glycylglycylglycine, and leucine amide correspond to the axes on the right; acetyltyrosine ethyl ester and benzoylarginine ethyl ester correspond to the axes on the left. The cathode is to the left.

Table 3. *Properties of some fractions from the electrophoresis experiment shown in Fig. 3*

Fraction number	13	17	18	20
Total N (mg. N/ml.)	0·1195	0·153	0·094	0·0765
$E^{1\,cm.}_{1\,mg.\,N/ml.}$ at 280 mμ.	8·2	9·7	—	5·82
Phosphatase activity (units/mg. N)	0	12 900	9930	0
Peptidase activity: glycylglycylglycine hydrolysis (min.$^{-1}$/mg. N/ml.)	0	$2·06 \times 10^{-2}$	$12·9 \times 10^{-2}$	0
Proteinase activity (units/mg. N)	5·9	0	0	0
Activity towards acetyltyrosine ethyl ester (μmole/hr./mg. N)	15·7	0	0	0

Minimum activity of pure phosphatase, and pure peptidase calculated from fractions 17 and 18, assuming that only these two enzymes are present: phosphatase, 13400 units/mg. N; peptidase, 0·50 min.$^{-1}$/mg. N/ml.

Table 4. *Influence of β-phenylpropionic acid (hydrocinnamic acid) on seminal proteinase and chymotrypsin*

The results are expressed as % of the uninhibited reaction velocity. The experiment with casein was performed in the usual way; the manometric experiments with acetyltyrosine ethyl ester were performed in 0·1 M-$NaHCO_3$, the gas phase contained 30% CO_2. Methanol not added. Concn. of hydrocinnamate, 0·08 M in all experiments.

Substrate	Seminal enzyme	Chymotrypsin (0·5 μg.)	Chymotrypsin (2·5 μg.)
Casein	55	83	81
Acetyl-L-tyrosine ethyl ester	167	750*	100

* For interpretation of this apparent activation, see text.

towards the group of simple peptides, the maximum of which was found a little to the right of the phosphatase peak, did not correspond to any apparent peak or shoulder on the curve. The relative position of these activities is in complete agreement with the findings by paper electrophoresis. The fibrinolysin activity was not determined in this experiment, but in other similar experiments it was situated on the left part of the proteinase peak.

Table 3 shows the relative purity of the three enzyme preparations from the fractions with maximum proteinase, phosphatase and peptidase activity. On the assumption that fractions 17 and 18 contain phosphatase and peptidase as the only proteins, the specific activities of these enzymes were calculated. The turnover number for the phosphatase was calculated to be 38 000 min.$^{-1}$, arbitrarily assuming the molecular weight to be 100 000. The purifications attained were: for proteinase, approximately 3 times; for phosphatase, 4·5 times; and for peptidase activity, approximately 10 times. The activity of fraction 18 towards benzoylarginine ethyl ester was 0·69 μmole/mg. N/min. Assuming that the same enzyme hydrolyses triglycine and benzoylarginine ethyl ester, the minimum activity is 2·7 μM/mg. N/min., which is about 10 times less than the activity of trypsin.

Properties of the proteinase. In all the enzyme preparations examined so far, whether crude ammonium sulphate precipitates or enzyme preparations purified by the various procedures described, we have always found a constant relation between the activities towards casein and acetyltyrosine ethyl ester, suggesting that the same enzyme is responsible for the two effects. As acetyltyrosine ethyl ester is characteristically hydrolysed by chymotrypsin, a comparison was made between the two proteolytic enzymes (Table 5).

The seminal proteinase has approximately the same pH optimum as chymotrypsin towards casein, but the pH/activity curve for chymotrypsin has a much broader maximum than the seminal enzyme (Northrop, Kunitz & Herriott, 1948). Benzoylarginine ethyl ester is hydrolysed very slowly by the seminal proteinase, and also by chymotrypsin as shown by Schwert *et al.* (1948). Kaufman & Neurath (1949) have found that hydrocinnamic acid is an effective inhibitor of chymotrypsin. This seems also to be the case with seminal proteinase when casein is used as the substrate. With acetyltyrosine ethyl ester no inhibition was observed with hydrocinnamate either with the seminal enzyme or with chymotrypsin. On the contrary a definite activation was observed in both cases, which was particularly marked with a low concentration of chymotrypsin (Table 4). With chymotrypsin the result may be due to a stabilizing effect of the hydrocinnamate, as chymotrypsin at the very low concentration used deteriorates rapidly. In this case then it is not possible to decide with certainty whether the addition of hydrocinnamate has any influence on the reaction velocity.

One difference between chymotrypsin and the seminal proteinase is, however, quite unambiguous, the seminal enzyme not being inhibited by di*iso*propyl phosphorofluoridate (DFP) at a concentration of 0·003 M, in which conditions chymotrypsin is completely inhibited. The seminal proteinase is distinguished from the so-called cathepsins by not requiring any activator, such as cyanide or cysteine, for full activity.

Properties of the seminal aminopeptidase. The enzyme which hydrolyses simple peptides such as alanylglycine, leucylglycine, glycylleucine, etc.,

Table 5. *Comparison between seminal proteinase and chymotrypsin*

	Seminal enzyme	Chymotrypsin
pH optimum (casein)	7·5	7–9
Hydrolysis of acetyl-L-tyrosine ethyl ester	Rapid	Rapid
Hydrolysis of benzoyl-L-arginine ethyl ester	Slow	Very slow
Coagulation of milk	+	+
Inhibition by di*iso*propyl phosphorofluoridate (0·003 M)	None	Complete
Cysteine (0·005 M)	No activation	No activation
Ethylenediaminetetraacetate (0·002 M)	No influence	No influence
β-Phenylpropionic acid (0·08 M)		
Substrate: casein	Inhibition	Some inhibition
Substrate: acetyl-L-tyrosine ethyl ester	Activation	(Activation?)

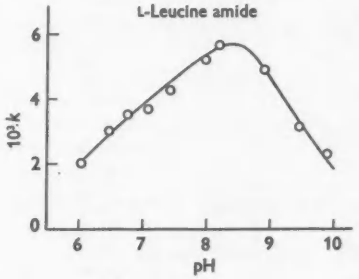

Fig. 4. The effect of pH on hydrolysis of L-leucine amide by crude seminal enzyme. The results are expressed as first-order velocity constants. Buffer: 0·02 M phosphate, temp. 37°.

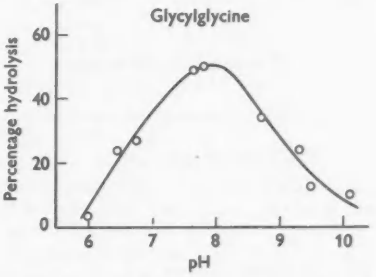

Fig. 5. The effect of pH on the hydrolysis of glycylglycine. Conditions as in Fig. 4. Results are expressed as percentage hydrolysis.

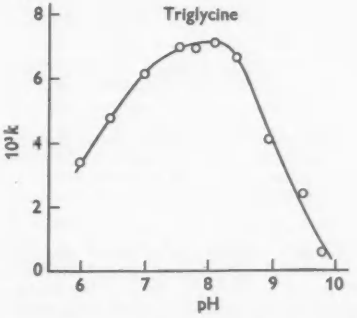

Fig. 6. The effect of pH on the hydrolysis of glycylglycylglycine. Experimental conditions as in Fig. 4.

apparently requires a free amino group. Apart from this condition we have, with the limited number of peptides available, found no special requirements regarding type or number of the amino acids present in the substrate. Among the substrates tested, alanylglycylglycine was found to be most readily attacked (Table 1). Leucine amide, which has been used as a substrate for leucine aminopeptidase (Smith & Slonim, 1948), is hydrolysed at a rate comparable with that of the other peptides used, and both glycylleucine and leucylglycine are hydrolysed.

The pH optimum for the hydrolysis of three different substrates (Figs. 4–6) was found to be around 7·8–8·0 and the general shape of the curves is similar in the three cases.

As nearly all aminopeptidases described in the literature require or are activated by various metal ions, this point was further investigated. Some illustrative experiments are collected in Table 6. No significant activation was observed with any of the metals used. The enzyme preparations as generally used were dialysed against distilled water for 30 hr. In order to remove tightly bound metals, an experiment was performed with additional dialysis against an acid buffer of pH 4·0 for a period of 30 hr. This treatment did not cause any decrease in enzymic activity, neither did dilution of the thoroughly dialysed enzyme with 4 vols. of water lower the velocity expressed on the basis of mg. N.

Addition of substances which bind metals through complex formation was also attempted. Cyanide and hydroxylamine had hardly any effect, but ethylenediaminetetraacetate had some effect in all cases, amounting to 50–70 % inhibition, dependent upon the substrate used (Table 6). The decrease in enzymic activity is, however, small compared with the effect observed with metal-requiring peptidases.

Table 6. *Influence of metal ions on the seminal aminopeptidase*

Hydrolysis measured by acetone titrations. pH 7·8, 37°. Results are expressed as first-order reaction constants.

Enzyme preparation	Additions	Concn. (M)	$10^3\,k$
	Substrate: L-leucine amide		
Crude enzyme	None	—	5·3
	Mn^{2+}	0·001	5·7
	Co^{2+}	0·001	5·0
Crude enzyme	None	—	3·3
	Mg^{2+}	0·001	3·1
	Zn^{2+}	0·001	2·0
	EDTA*	0·001	1·8
	EDTA	0·002	1·5
	EDTA + Mg^{2+}	0·002†	2·0
	EDTA + Zn^{2+}	0·002†	2·1
Fraction from electrophoresis	None	—	1·09
	EDTA	0·001	0·82
	EDTA + Fe^{2+}	0·002†	0·76
	Substrate: glycylglycine		
Crude enzyme	None	—	1·25
	Fe^{2+}	0·001	1·11
	Mn^{2+}	0·001	1·05
	EDTA	0·001	0·47
	EDTA + Fe^{2+}	0·002†	1·12
	EDTA + Mn^{2+}	0·002†	0·72
Crude enzyme	None	—	0·88
	Co^{2+}	0·001	1·30
	EDTA	0·001	0·32
	EDTA + Co^{2+}	0·002†	0·19
	Substrate: glycylglycylglycine		
Fraction from electrophoresis	None	—	3·1
	EDTA	0·001	1·6
	EDTA + Fe^{2+}	0·002†	1·1
Crude enzyme	None	—	11·3
	EDTA	0·001	3·7
	EDTA + Mg^{2+}	0·002†	2·8

* Ethylenediaminetetraacetate. In all experiments where this substance has been added with metal ions, the concentration was 0·001M. In experiments with EDTA addition the reaction rate (first-order constant) decreased during the experiment. The values listed are averages.

† Concentration of metal ions.

Table 7. *Hydrolysis of amino acid esters by seminal enzyme*

Manometric experiments in bicarbonate buffer, pH 7·7, 35°. The results are corrected for spontaneous hydrolysis.

Substrate	μmole/hr./ml.
Benzoyl-L-arginine ethyl ester	13·5
L-Leucine ethyl ester*	1·4
L-Cysteine ethyl ester	0
N-Phenylglycine ethyl ester	0
Acetyl-DL-alanine ethyl ester	0

* The spontaneous hydrolysis of leucine ethyl ester was appreciable, viz. 1·2 mole/hr./ml.

Attempts to reverse the inhibition by addition of an excess of metal ions were negative in all cases except where glycylglycine was used as substrate, where addition of ferrous iron brought about a fairly complete reversal. This observation may suggest the presence of a certain amount of an iron-activated glycylglycine dipeptidase, but apart from this finding no experimental support for the assumption that two peptidases might be present has been found so far. The possibility that ethylenediaminetetraacetate may have an inhibitory effect *per se*, which is not due to complex formation with activating metals, should perhaps be taken into account. In these experiments the substances added (metal salts and EDTA), were incubated with the enzyme for 1 hr. before addition of substrate.

The enzyme which hydrolyses benzoyl-L-arginine ethyl ester. The enzyme in human semen which catalyses hydrolysis of the ester bond in benzoyl-L-arginine ethyl ester is apparently not identical with trypsin or an enzyme resembling trypsin, as in the fractionation experiments the esterase was completely separated from any enzyme causing casein

Table 8. *Inhibition of the enzyme which hydrolyses benzoyl-L-arginine ethyl ester and of thrombin by diisopropylphosphorofluoridate*

Enzyme	DFP (M)	Activity (μmole/hr.)	Average	Percentage activity
1·5% soln. of freeze-dried enzyme (0·8 ml.). Buffer: 0·1 M-$NaHCO_3$; gas phase: 30% CO_2+70% N_2; pH 7·4; 35°	0	5·53 5·33	5·43	100
	0·0015	2·91 2·98	2·95	54
	0·003	2·74 2·90	2·82	52
Same enzyme (0·8 ml.). Buffer: 0·4 M-$NaHCO_3$; gas phase: 5% CO_2+95% N_2; pH 7·7; 35°	0	8·67 8·63	8·65	100
	0·003	4·45 4·47	4·46	51·5
Thrombin. Buffer: 0·04 M-$NaHCO_3$; gas phase: 5% CO_2+95% N_2; pH 7·7; 35°	0	8·95 9·00	8·98	100
	0·003	0	—	0

Table 9. *Attempts to differentiate between aminopeptidase and the enzyme hydrolysing benzoyl-L-arginine ethyl ester*

The results are expressed as a percentage of the activity without any treatment.

Treatment	Benzoylarginine ethyl ester	Glycylglycyl-glycine	Leucine amide
None	100	100	100
EDTA 0·001 M	100	50	50
Heating at pH 5·5 to 60° for			
8 min.	10·5	75	—
16 min.	7·6	44	—
DFP 0·003 M	52	—	88

hydrolysis. Furthermore, no inhibition was observed by purified soybean inhibitor in amounts sufficient to completely inhibit pure trypsin in control experiments.

In electrophoresis experiments the esterase followed closely the aminopeptidase, so closely indeed that there seemed to be a constant relationship between the two activities, and all attempts to separate them have so far been unsuccessful.

A number of amino acid esters were tried as substrate for this enzyme (Table 7). Apart from benzoyl-L-arginine ethyl ester, only L-leucine ethyl ester was hydrolysed at a measurable rate.

Heating at pH 5·5 to 60° causes a more rapid decrease in the activity towards the benzoylarginine ethyl ester than towards the simple peptides such as triglycine and leucine amide (compare Table 9). This finding definitely points to the possibility that two different enzymes are responsible for the peptidase and the esterase effect. Experiments with DFP revealed that the esterase was inhibited to 50% both at 0·001 and 0·003 M-DFP, whereas the aminopeptidase was hardly inhibited at all. In order to exclude the possibility that hydrolysis of the DFP caused a significant change in the pH, experiments were also performed with high buffer concentration (Table 8).

Troll, Sherry & Wachman (1954) have found that plasmin hydrolyses benzoylarginine ethyl ester, and Sherry & Troll (1954) have observed that thrombin also has a very marked ability to hydrolyse this ester. In our experiments plasmin can be excluded in advance, as no trace of fibrinolytic activity was present in fractions rich in the enzyme which splits benzoylarginine ester. The hypothesis that thrombin is responsible for the hydrolyses was tested by comparing thrombin solutions with those fractions from an electrophoresis experiment displaying the highest activity in the benzoylarginine ethyl ester assay. Thrombin in amounts hardly measurable with the manometric technique still showed good activity in the clotting test with human oxalated plasma, whereas even the most active fractions of seminal enzyme did not clot human plasma. Finally, thrombin was found to be completely inhibited by DFP (Table 8). The evidence suggesting that aminopeptidase and the enzyme which hydrolyses benzoylarginine ester are different enzymes is summarized in Table 9.

The fibrinolytic enzyme. The factor in our crude enzyme preparations which causes lysis of fibrin clots both in tubes and with the plate method was considerably purified by the calcium phosphate fractionation procedure. No fibrinolytic reaction was observed when enzyme preparations (both crude and purified) were applied to fibrin plates previously heated to 90°, in order to destroy plasminogen, which is always present in fibrinogen. Control experiments with trypsin showed that the fibrin plates retained most of their susceptibility to trypsin (about 70%). This indicates that the fibrinolytic activity in our preparations is probably due to a plasminogen activator similar to fibrinokinase. The possibility should, however, be kept in mind that the heat treatment may have rendered

the fibrin coagulum resistant towards the enzyme in semen.

The site of formation of the seminal enzymes. A number of experiments have been performed with prostate and vesicular gland tissue removed at autopsy from male human corpses within 24 hr. after death. Both aminopeptidase and proteinase were found to be present in high concentrations in prostate tissue and only in small amounts, if at all, in vesicular gland extracts.

DISCUSSION

Among the enzymes studied there seems to be none that causes lysis of fibrin directly. Neither have we found any precursor of fibrinolytic enzymes, but only what appears to be an activator. The work of von Kaulla & Shettles (1953) nevertheless indicates that both a precursor, a kinase, and consequently a fibrinolytic enzyme are present in human seminal fluid. The failure to demonstrate the presence of any active enzyme may be due to the fact that all our preparations were made by ammonium sulphate fractionation. The precipitate formed at 40% saturation, which was discarded, may have contained the true fibrinase. Another possibility is that this enzyme is precipitated and consequently discarded during the dialysis against distilled water, which is also a step common to all our enzyme preparations.

The proteinase described, which was found in earlier experiments to attack fibrinogen among a number of proteins (Lundquist, 1952), may be identical with the fibrinogenase of Huggins & Neal (1942). The function of this enzyme in the ejaculate is probably to break down further the large fragments formed by the action of the fibrinolytic enzyme system. The final step in this scheme would then logically be the formation of free amino acids through the action of the aminopeptidase.

The seminal aminopeptidase does not appear to be identical with any of the peptidases described in the literature (see, for example, the review by Smith, 1951). It hydrolyses dipeptides as well as tripeptides, and shows no preference for leucylpeptides. The fact that no metal activation was observed also distinguishes this enzyme from the majority of peptidases. Certain cathepsins with ability to split small peptides do not require metal activation, but need activation by reducing substances, such as cysteine or cyanide. These substances have no effect on the seminal peptidase. However, the possibility cannot be excluded that we are dealing with two or more enzymes of very similar physicochemical properties, which we have not succeeded in separating; the fact that the relative rates of hydrolysis for a number of substrates were constant throughout all fractionation procedures speaks rather against this possibility.

Purification of prostate phosphatase has been reported by London & Hudson (1953). Their method appears to be rather laborious, involving fractionation with acetone, ammonium sulphate, calcium phosphate absorption and 'foam fractionation'. They claim to have found a turnover number for their purest preparation of 100000. A simple and apparently very efficient method for the purification of this enzyme by chromatography on ion-exchange resins has recently been described by Boman (1954).

Roche & Bouchilloux (1953) have reported the preparation of electrophoretically homogeneous alkaline intestinal phosphatase. From their results a turnover number of 23000 is calculated, based on an assumed molecular weight of 100000. They use glycerophosphate as substrate, and when it is taken into account that phenylphosphate is hydrolysed about twice as rapidly (King & Delory, 1939), the turnover number found by the French workers is not very different from that calculated for our seminal enzyme preparations. This finding may suggest a considerable similarity in the catalytic activity of acid and alkaline phosphatases.

SUMMARY

1. By means of various fractionation methods, including adsorption on tricalcium phosphate gel and zone electrophoresis on starch, the separation of at least two proteolytic enzymes from human seminal plasma was accomplished.

2. One of these is a proteinase with a certain resemblance to chymotrypsin. It hydrolyses acetyltyrosine ethyl ester, it does not require metal activation or activation by reducing substances. It differs from chymotrypsin in not being inhibited by di*iso*propylphosphorofluoridate and in the shape of the pH/activity curve.

3. The other proteolytic enzyme is an aminopeptidase with a pH optimum at 7·8 (tested with three different substrates). It does not require metal ions and hydrolyses both di- and tri-peptides. There seems to be no preference for leucylpeptides.

4. An enzyme was found which hydrolyses benzoylarginine ethyl ester very rapidly. It differs from the aminopeptidase in heat stability and in sensitivity towards di*iso*propylphosphorofluoridate and it is not inhibited by ethylenediaminetetraacetate. This enzyme was not separated from aminopeptidase and is not identical with trypsin, thrombin or plasmin.

5. A fibrinolytic factor, which appears to be similar to fibrinokinase, was separated from other enzymes. Considerable purification of this factor was accomplished by adsorption on tricalcium phosphate.

6. Prostate phosphatase was prepared in high purity by means of zone electrophoresis. A turnover

number of 38 000 was calculated for the purest preparation.

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The Effect of *o*-Phenylenediamine on the Biosynthesis of Vitamin B_{12}: A New Vitamin B_{12} Analogue

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By adding *o*-phenylenediamine or any one of a number of related substances to a synthetic medium, Dulaney & Williams (1953) claimed to have doubled or trebled vitamin B_{12} production by *Streptomyces griseus* and some other species, as judged by the *Escherichia coli* (mutant) assay. 1:2-Diamino-4:5-dimethylbenzene, a part of the vitamin B_{12} molecule and a postulated precursor of it (Woolley, 1950, 1951), had no such effect. These authors thought it unlikely that increased vitamin B_{12} was produced by incorporating phenylenediamine into the molecule. Tracer studies with an unstated level of deutero phenylenediamine showed that not more than 20% of the vitamin was labelled. It was shown that phenylenediamine inhibited the growth of *Strep. griseus* and it was suggested that a compound antagonistic to phenylenediamine, namely vitamin B_{12}, was produced in increased amounts so as to overcome this inhibition.

We thought that the apparent increased vitamin B_{12} titre might be caused by a different mechanism and therefore re-investigated the problem. A preliminary account of this work has already been published (Fantes & O'Callaghan, 1954).

METHODS

Medium. The synthetic medium used was the same as that described by Dulaney & Williams (1953). It was autoclaved at 15 lb./sq.in. steam pressure for 20 min. Phenylenediamine (autoclaved separately as the hydrochloride) was added to the medium just before inoculation. Varying concentrations of phenylenediamine were prepared in such a way that the addition of the same volume (1 ml.) produced the desired level of phenylenediamine in the medium.

Growth conditions. The strain of *Strep. griseus* used was derived from the same parent strain as the one used by Dulaney & Williams, but was not identical with it. The preparation of the vegetative inoculum and the fermentations were again done essentially as described by the American authors, except that 40 ml. medium were used/250 ml. flask. Instead of autoclaving the diluted samples before assaying, they were heated in a boiling-water bath for 10 min.

Microbiological assay. Two types of microbiological assay were employed: an *Esch. coli* plate assay (Harrison, Lees & Wood, 1951), but with the medium devised by Burkholder (1951), and a *Lactobacillus leichmannii* plate assay.

Chromatography and bioautographs. For the chromatographic analysis equal volumes (about 10 μl.) of the heated samples were applied to Whatman no. 4 sheets. Development was downward with butanol nearly saturated with water (98 parts of saturated and 2 parts of dry solvent). The bottom of the tank was covered with the same solvent mixture and the inside lined with gauze, which dipped into the liquid covering the bottom of the tank. A small beaker with aqueous KCN was also kept in the tank to prevent the spontaneous conversion of the cyano into the hydroxo compounds. Development was carried out at room temperature for 24–48 hr.

Bioautographs were prepared by a technique similar to the one first described by Cuthbertson & Lester Smith (1949), but making use of the improvements introduced by Ford & Holdsworth (1953). For a quantitative assessment of relative zone sizes the plates were photographed after incubation; the white zones were then cut out from the photographs and weighed. Three or four levels of pure vitamin B_{12} were chromatographed on the same sheet, when quantitative estimations were desired. It was found that the zone weight was proportional to the logarithm of the amount of vitamin applied to the paper. Graphs were constructed and the amount of vitamin B_{12} in experimental samples was ascertained by means of them. (The weighing of zone photographs as a means of estimating zone size was suggested to us by Mr G. C. Ashton of these laboratories.)

RESULTS

A typical culture containing 40–480 μg. *o*-phenylenediamine base/ml. of medium was assayed on the fourth, fifth and sixth day by the *Esch. coli* and *Lb. leichmannii* plate assays. The results obtained on the fourth day are shown in Table 1; the results obtained on the subsequent days were similar.

The table shows clearly that certain concentrations of phenylenediamine in the medium produced titres far in excess of the controls, although higher concentrations of phenylenediamine considerably inhibited growth. In another experiment the contents of some of the flasks and four levels of vitamin B_{12} were subjected to chromatographic analysis and bioautography with both test organisms. All samples containing phenylenediamine showed the presence of a new substance with an R_F value just below that of vitamin B_{12}. The zone size of this new growth factor increased with increasing levels of phenylenediamine, the zones due to vitamin B_{12} decreasing simultaneously. When the weights (78·0, 63·6, 54·2, 42·0 mg.) of the zones due to the four vitamin B_{12} standards were plotted against the logarithms of their concentrations (0·4, 0·2, 0·1, 0·05 μg./ml.) a straight line was obtained. From the vitamin B_{12} zone weights of the samples their true vitamin B_{12} content could then be deduced from the graph. The results are shown in Table 2.

Although the presence of phenylenediamine in the medium gave rise to increased 'vitamin B_{12}' titres, biosynthesis of true vitamin B_{12} was depressed.

Table 1. *The effect of* o*-phenylenediamine on vitamin* B_{12} *titre of* Streptomyces griseus *fermentation liquor (day* 4)

Each figure represents the average assay value obtained from three individual shake flasks.

Concentration of *o*-phenylenediamine (μg./ml. medium)	0	40	80	160	240	320	480
μg. apparent vitamin B_{12}/ml. (*Esch. coli*)	0·22	0·41	0·50	0·55	0·52	0·39	0·29
μg. apparent vitamin B_{12}/ml. (*Lb. leichmannii*)	0·22	0·40	0·42	0·37	0·37	0·32	0·25

Table 2. *Comparison of vitamin* B_{12} *content of* Streptomyces griseus *fermentation liquor obtained by direct assay and calculated from chromatographic zone size*

Concentration of *o*-phenylenediamine (μg./ml. medium) ...	0	80	160	320
Apparent vitamin B_{12} content (μg./ml., *Esch. coli* assay)	0·36	0·48	0·79	0·55
True vitamin B_{12} content (μg./ml., obtained by chromatography and *Esch. coli* bioautography)	0·36	0·069	0·050	0·033

To find out if the formation of the new growth factor could be suppressed by the inclusion in the medium of 1:2-diamino-4:5-dimethylbenzene, fermentations were carried out with a level of 80 μg. phenylenediamine/ml. and various concentrations of diaminodimethylbenzene, the lowest being 2 μg./ml. Bioautographs showed the complete absence of the new factor in these experiments. In order to determine the 'inhibition index' a further experiment was carried out with 40 μg. phenylenediamine/ml. and 2, 1, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{80}$ μg. diaminodimethylbenzene/ml. Only vitamin B_{12} was formed in the presence of 2, 1, $\frac{1}{2}$, $\frac{1}{4}$ μg. diaminodimethylbenzene/ml. With $\frac{1}{8}$ μg. diaminodimethylbenzene/ml. a small amount of the new material was produced; $\frac{1}{80}$ μg. diaminodimethylbenzene/ml. gave zones similar to a control containing only 40 μg. phenylenediamine/ml. The inhibition index in this system therefore lies somewhere between 160 and 320.

Isolation of new factor

As isolation of the new substance from the synthetic medium was difficult, we investigated the effect of phenylenediamine on a complex medium capable of much higher productivity. Qualitatively the picture was similar, although the total 'vitamin B_{12}' titre achieved in the presence of phenylenediamine was only slightly above that in its absence. Purification procedures normally used for isolating vitamin B_{12} were applied to a fermentation broth that initially contained 160 μg. phenylenediamine/ml. A red concentrate from it contained vitamin B_{12} and the new substance. This was streaked on to Whatman 3 MM sheets and chromatographically developed in the same way as described above for bioautograph samples. Two red bands formed. The ones containing the new substance were cut out and eluted with water; the eluate was concentrated and the solute crystallized from aqueous acetone. Its spectrum strongly resembled that of vitamin B_{12} and had a similar main peak at 361 mμ.

Microbiological activity of new factor

The microbiological potency of the new substance was compared with that of vitamin B_{12}. Solutions of the two materials with equal optical densities at 361 mμ. were prepared and appropriate dilutions were assayed on *Esch. coli* and *Lb. leichmannii* plates. Under these conditions the new substance was approximately 1·8 times as active as vitamin B_{12} for *Lb. leichmannii* and 2·4 times as active for *Esch. coli*.

Partial identification of the new factor

The crystalline material (3·0 mg.) dissolved in 6N-HCl (1·5 ml.) was heated at 150° in a sealed tube for 7·5 hr. (Buchanan, Johnson, Mills & Todd, 1950). The yellow-brown solution was freed from a little insoluble material by filtration and from excess hydrochloric acid by concentration under reduced pressure. The residue, dissolved in a few ml. water, was passed through an 'Amberlite' IR-120 (H^+) resin column (6 × 1 cm.), which was then washed with water (100 ml.). 2N-NH_3 (100 ml.) was next passed through the column, the eluate was taken to dryness *in vacuo* and the residue dissolved in very little 0·1N-HCl; several spots of it were applied to a filter paper sheet (Whatman no. 4). Benziminazole and 5:6-dimethylbenziminazole were used as standards; chromatography and detection of fluorescent spots was carried out as described by Buchanan *et al.* The hydrolysate and benziminazole gave spots with an R_F of 0·70; the R_F of the dimethylbenziminazole was 0·74. The hydrolysate showed a further small spot with an R_F of 0·82. The hydrolysate spots were cut out and eluted with 0·1N-HCl and the absorption spectra between 200 and 300 mμ. were determined. The slower moving substance revealed two maxima (273·5 and 267 mμ.) in the same positions as those of benziminazole (5:6-dimethylbenziminazole has maxima at 283 and 275 mμ.). The spectrum of the faster moving substance showed no maximum between 250 and 300 mμ. and only general absorption at wavelengths below 250 mμ. This spot could perhaps have arisen from an impurity.

DISCUSSION

The increased apparent vitamin B_{12} titre claimed by Dulaney & Williams (1953) when *o*-phenylenediamine was added to the medium of *Strep. griseus* fermentations is not due to a higher production of this vitamin itself, but to the formation of a vitamin B_{12} analogue. This probably differs from the former only in lacking the two methyl groups of the benziminazole portion. The new material is microbiologically more active than vitamin B_{12} (in plate assays). Its enhanced activity is probably the only reason for the observed titre increases in fermentations containing phenylenediamine, though it is not impossible that phenylenediamine may also have stimulated somewhat the synthesis of the main cobalt containing portion (factor *B*, Ford & Porter, 1952; Gant, Lester Smith & Parker, 1954), common to both substances. If such a stimulation does take place, then a mechanism similar to the one suggested by Dulaney & Williams (1953) may well be operative though the biosynthesis of a portion only of the vitamin B_{12} molecule, and not of the whole, would be involved.

The increasing levels of the new substance at the cost of vitamin B_{12} with increasing levels of phenylenediamine in the medium, and the complete absence of biosynthesis of the new substance in the

simultaneous presence of phenylenediamine and relatively low levels of diaminodimethylbenzene, makes it most probable that a mechanism of competition is operative. Whether the actual synthesis of diaminodimethylbenzene (or a more complex derivative of it) is suppressed by phenylenediamine, or whether diaminodimethylbenzene is synthesized at its normal level irrespective of the presence of phenylenediamine, but not incorporated into the vitamin B_{12} molecule if excess phenylenediamine is present is not known.

The new substance is almost certainly identical with the one obtained independently by Ford, Holdsworth & Kon (1954), who incubated *Esch. coli* and factor *B* in the presence of benziminazole and then demonstrated by chromatography the formation of a new compound with an R_F value just less than that of vitamin B_{12}.

SUMMARY

1. Fermentations of *Streptomyces griseus* on a complex or a synthetic medium in the presence of *o*-phenylenediamine gave rise to the production of a new analogue of vitamin B_{12}.

2. The new substance has been isolated in the form of red needles.

3. Its absorption spectrum is practically identical with that of vitamin B_{12}.

4. Benziminazole has been identified as a hydrolytic product.

5. The new material is microbiologically (plate assay) more active than vitamin B_{12}: the greater activity probably explains the apparently increased 'vitamin B_{12}' titres obtained by Dulaney & Williams (1953).

6. The biosynthesis of true vitamin B_{12} is competitively inhibited by phenylenediamine.

7. The biosynthesis of the new material in the presence of phenylenediamine is competitively inhibited by 1:2-diamino-4:5-dimethylbenzene. The inhibition index lies somewhere between 160 and 320.

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Note added in proof. Clinical trials kindly carried out by Professor L. J. Witts and Dr Sheila Callendar at the Radcliffe Infirmary, Oxford, showed that the new material was fully active in three pernicious anaemia patients.

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The Vitamin B_{12} Group. Presence of 2-Methyl Purines in Factors *A* and *H* and Isolation of New Factors

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The main cobalt-containing substance in the rumen content and faeces of calves has been called 'factor *A*' by Ford & Porter (1952, 1953). The absorption spectrum and microbiological activity suggested its close relation to vitamin B_{12}. Preliminary reports showed that in fact the difference lies only in replacement of the 5:6-dimethylbenziminazole of vitamin B_{12} by 2-methyladenine, a purine not previously found in nature (Brown & Lester Smith, 1954; Gant, Lester Smith & Parker, 1954). Dion, Calkins & Pfiffner (1954) have independently characterized as 2-methyladenine the base present in factor *A*, which they call pseudo (ψ)-vitamin B_{12d}.

By extracting portions totalling 170 kg. of pig manure and about 180 kg. of calf manure, we were able to isolate not only factor *A* and vitamin B_{12} itself but also small amounts of other factors, namely, ψ-vitamin B_{12} (Dion, Calkins & Pfiffner, 1952), factors *B* and *C* (Ford & Porter, 1952) and several new red substances that we call factors *D–I*. Some of them were separated only by employing both chromatography and electrophoresis on paper, successively but in either order. Factors *G* and *H* could not be separated except by taking advantage of slight differences in the behaviour of their purple dicyano complexes. Factor *I* appears identical

with 'factor III' of Friedrich & Bernhauer (1953). The chemical relationships between some of these factors have been revealed by characterizing the products of their hydrolysis under various conditions.

EXPERIMENTAL

Isolation of factors

The methods used were adapted, as the work proceeded, from those used for the isolation of vitamin B_{12} from liver and fermentation liquors (Fantes, Page, Parker & Lester Smith, 1949). Close collaboration was maintained with workers at the National Institute for Research in Dairying, who have already reported typical procedures (Ford & Porter, 1953).

In brief outline the process comprised the following steps. The manure as received was extracted with boiling ethanol (about 50%, v/v, allowing for water present in the material) containing a trace of cyanide, filtered with kieselguhr, concentrated under reduced pressure, filtered after adjustment to pH 7 and addition of ethanol to 60% (v/v), and concentrated under reduced pressure. It was adsorbed by percolation through a bed of charcoal, the adsorbate was washed with 6% (v/v) aqueous phenol, eluted with hot 60% (v/v) aqueous acetone, and concentrated under reduced pressure. Impurities were then removed by passage through a column of acid-washed alumina, which was developed with water.

Partition chromatography was used for the final stages of purification and for partial separation of the factors. At first this operation was carried out on columns of kieselguhr containing half its weight of water, with water-saturated *n*-butanol for development. Later it was found preferable to streak the partially purified extract across sheets of Whatman 3 MM filter paper and to develop downwards for 24–48 hr. at 27° in the dark, with water-saturated *sec.*-butanol; a small beaker of aq. HCN was present in the tank to keep the factors in their cyano-coordination states. The solvent front ran off the bottom of the paper (serrated with pinking shears to ensure even flow), so that R_F values can only be quoted relative to that of a marker spot of vitamin B_{12}. Red zones were eluted and further fractionated by electrophoresis on Whatman no. 2 filter paper or Whatman seed-test paper in 0·5N acetic acid (pH 3·0) as supporting electrolyte (Armitage *et al.* 1953; Holdsworth, 1953). A voltage gradient of about 5v/cm. for about 16 hr. usually sufficed to produce distinct zones of the basic factors (*A*, *B* and ψ-vitamin B_{12}), separate from each other and from the group of neutral factors. For special purposes electrophoretic separations under similar conditions were carried out with other electrolytes, namely 0·05M sodium phosphate buffer containing 0·01% KCN at pH 6·5 or 7·5, or 0·05M-KCN (pH 10 approx.). Repetition of either chromatography or electrophoresis or both was necessary to ensure complete removal of other factors; identification of the known factors was simultaneously accomplished by running spots of authentic specimens alongside.

The earlier stages of fractionation were controlled by microbiological plate assays with the B_{12}-requiring *Escherichia coli* mutant (Harrison, Lees & Wood, 1951) and also by chromatography and electrophoresis of small amounts on filter paper, the positions of the microbiologically active factors (i.e. all except factor *D*) being revealed by the bioautographic technique (Ford & Holdsworth, 1953). The purified factors were also assayed with the more selective micro-organisms *Lactobacillus leichmannii* (Ford, 1953*a*) and *Ochromonas malhemensis* (Ford, 1953*b*).

Factor *D* was seen as a slow-running pink band on chromatographing partially purified calf-manure extracts on alumina from aqueous solution. It was microbiologically inactive and came through the column after all the active fractions had been eluted. Factor *D* was purified further by paper chromatography and obtained as a deep red solid that could not be induced to crystallize. It behaved as a base on electrophoresis in acetic acid. The absorption spectrum suggests a close relationship to the B_{12} vitamins, of which it is perhaps a metabolic product.

The other factors are described briefly in the order in which they separate on paper chromatography; some of their properties are given in Table 1.

Factor *C* (Ford & Porter, 1952; Holdsworth, 1953) was revealed on bioautographs of both source materials as the slowest chromatographic zone (R_F relative to vitamin B_{12}, about 0·2). The zone often appeared as a doublet, suggesting the presence of two factors. Electrophoresis in neutral or alkaline buffers revealed the acidic character of this factor (or factors). Purification proved exceedingly difficult and so far only brown concentrates have been obtained from these sources.

Factors *A*, *G*, *H* and ψ-vitamin B_{12} appeared together on paper chromatograms with R_F values (relative to vitamin B_{12}) of 0·35–0·45; the R_F values appeared to be affected by impurities present in the concentrates. On paper electrophoresis in acetic acid, the mixture could be partially resolved. Factors *G* and *H* remained together near the origin, while factor *A* and ψ-vitamin B_{12} moved towards the cathode, factor *A* being the faster moving and more basic of the two.

Factors *G* and *H* proved more difficult to separate than any other members of this group so far encountered. Indeed, the factors themselves behaved identically on chromatography or electrophoresis under all the conditions tested. However, like cyanocobalamin itself, these factors take up an additional molecule of cyanide, signalled by a colour change to purple, in alkaline cyanide solutions. These acidic dicyano complexes of factors *G* and *H* could be separated by paper chromatography in an atmosphere containing HCN using *sec.*-butanol containing 0·2% of NH_3 (sp.gr. 0·88) as developing solvent, when factor *H* travelled slightly the faster. The purple complexes could not be separated by electrophoresis in 0·05M-KCN at pH 10, but separation did occur with 0·05M sodium phosphate buffer containing cyanide at pH 7·5.

At this pH, factor *H* was more completely converted into the acidic double cyanide than was factor *G*, so that its effective mobility was slightly greater: correspondingly, the colour of the zone was purplish mauve, whereas that of factor *G* was pinkish mauve.

Factor *I* appeared as a red zone with R_F (relative to B_{12}) of 0·65, on chromatographing both calf- and pig-manure concentrates. It was electrophoretically neutral. This factor appeared to be identical with 'factor III' isolated by Friedrich & Bernhauer (1953) from sewage sludge. We are indebted to Professor Bernhauer for a reference sample of their crystalline preparation. Clinical trials in Germany showed that it was active in pernicious anaemia. In clinical trials kindly carried out by Dr Sheila Callender at the Radcliffe Infirmary, Oxford, the German preparation gave an optimum response at 50 μg. while our factor *I* from pig manure gave a suboptimum response at 25 μg.

Factor *F* was found in manure from pigs, but only in traces from calf manure; it appeared to be the main factor, however, in chicken faeces. Its R_F value (relative to B_{12}) was 0·77; it was electrophoretically neutral.

Factor *E* appeared below the vitamin B_{12} zone, with a relative R_F of 1·35–1·5. It did not move from the origin on electrophoresis in acetic acid, but in phosphate buffer at pH 6·5 it moved towards the anode, and separated into two red zones of slightly different mobilities.

Factor *B* moved the furthest on chromatography, with relative R_F 1·6–1·7. On electrophoresis in acetic acid it moved towards the cathode, but faster than the other basic factors, *A* and ψ-vitamin B_{12}. Factor *B* differs from vitamin B_{12} only in the absence of the nucleotide portion (Armitage *et al.* 1953).

An alternative fractionation scheme followed for part of the material comprised electrophoresis in acetic acid as the first step yielding four fractions, namely (in order of separation from the cathode end) factor *B*, factor *A*, ψ-vitamin B_{12} and all the neutral factors. These last were then separated from one another by paper chromatography with the ammoniacal solvent mixture.

Factors *A*, *F*, *G*, *H*, *I* and ψ-vitamin B_{12} were obtained crystalline from aqueous acetone. It is hoped to report in more detail later, upon factors *C*, *D*, *E*, *F* and *I*.

Deamination

Factor *A* (4·2 mg.) was dissolved in water (1 ml.) and glacial acetic acid (0·05 ml.). The solution was held at 75° for 15 min., $NaNO_2$ (2 portions of 0·02 g.) being added initially and after 7·5 min. respectively. The warm solution was held briefly at reduced pressure to remove nitrous fumes and was then extracted with a few drops of phenol. The washed phenol extract was shaken with a few drops of water and 2 ml. of ether. Acetone was added to the aqueous layer until it became faintly turbid; crystals were deposited on keeping overnight. ψ-Vitamin B_{12} was deaminated similarly on a smaller scale.

Hydrolysis: isolation of purine base and preparation of picrate

Factor *A* (1·3 mg.) was dissolved in 2N-HCl (1·5 ml.) and the solution was heated under reflux in a boiling-water bath for 2 hr. The red hydrolytic products were removed by extraction with *n*-butanol (3 × 1 ml. portions), the extracts being washed in turn with 0·5 ml. of N-HCl. The aqueous layers were taken to dryness under reduced pressure to remove acid; the residues from this and similar hydrolyses were used for chromatography, electrophoresis and absorption spectroscopy. Part of the material was converted into the picrate by adding a slight excess of picric acid; the precipitate was collected by centrifuging and recrystallized from hot 25% (v/v) acetic acid. The infrared absorption spectrum in Nujol mull was compared with that of adenine picrate.

Other factors were hydrolysed similarly.

Hydrolysis: isolation of factor B

Factor *A* (1·2 mg.) was dissolved in conc. HCl (0·1 ml.) and the solution was held at 65° for 4·5 min. Acid was removed by evaporation under reduced pressure (bath temperature 60°) and the residue was dissolved in 1% KCN (0·5 ml.). The solution was acidified after 5 min. standing and extracted with phenol–benzene mixture (1:2, by vol.; 2 portions of 0·5 ml.). The extracts were shaken with a few drops of water and ether–acetone mixture (1:1, by vol.; 2 ml.). The red material in the aqueous layer was compared with a preparation made similarly from the same weight of vitamin B_{12}; the two alongside were submitted to paper chromatography and electrophoresis and to the microbiological plate assay with *Esch. coli* mutant, as described above.

Chromatography of purines

The purine bases released on hydrolysis with hot 2N-HCl as described earlier were chromatographed alongside appropriate 'markers' by descending development on Whatman no. 4 paper. After preliminary trials, two solvent systems were selected. The first consisted of *n*-butanol–NH_3 (sp.gr. 0·88) (97:3, by vol.) with just enough water to saturate the mixture. The second contained *n*-butanol–diethylene glycol–water (4:1:1, by vol.); an ammonia atmosphere was provided by a small beaker of NH_3 (sp.gr. 0·88) in the tank (Vischer & Chargaff, 1948).

RESULTS AND DISCUSSION

A property of factor *A* that proved invaluable in the course of its purification was its slight mobility towards the cathode during filter-paper electrophoresis in dilute acetic acid. This indication of a weakly basic group was confirmed by treatment with nitrous acid, which converted factor *A* into a different substance which was red and could be crystallized; this material, although it could not be distinguished from factor *A* by chromatography with *sec.*-butanol, was electrophoretically neutral. This behaviour was consistent with deamination of an amino group in the original substance. Moreover, the 'semi-synthetic' product appeared to be identical with a natural factor we have isolated in trace amounts from pig manure and call 'factor *H*' (Table 1). It is not necessarily synthesized directly by rumen micro-organisms, for it may arise by enzymic deamination of factor *A*. Hydrolysis of both factors, *A* and *H*, with hot 2N-HCl yielded (among other products) substances that appeared to be purines, for on chromatography they gave spots that quenched the fluorescence of the filter paper in ultraviolet light at 254 mμ., and they responded to staining reactions with cuprous ions (Holdsworth, private communication) or with silver chromate (Reguera & Asimov, 1950).

However, the R_F values in some solvent mixtures differentiated them from known natural purines (Table 2). Moreover, the purine from factor *A* displayed characteristic ultraviolet absorption maxima in neutral, acid and alkaline solution. Consideration of R_F values and electrophoretic mobilities suggested that this purine might well be an alkyl adenine, while that from the deaminated factor *A* could be the corresponding alkyl hypoxanthine. A step in the biogenesis of purines is

completion of the pyrimidine ring by reaction of the precursor 4-aminoiminazole-5-carboxamide with 'formate' (Buchanan & Wilson, 1953). It seemed possible that the abundant acetic acid in the rumen might partially displace formate from this role, leading to 2-methyl purines. We were fortunate to obtain synthetic specimens of 2-methyladenine and 2-methylhypoxanthine from Sir Alexander Todd, F.R.S.: by all the tests we were able to apply to quantities of less than 1 mg., their properties tallied with those of the bases from factors *A* and *H* respectively. In addition to those recorded in Table 2, the infrared absorption spectra of the crystalline picrates of 2-methyladenine and of the base from factor *A* in Nujol mull showed numerous corresponding peaks, in positions different from those given by adenine picrate.

The chromatograms from the hydrolysate of factor *A* showed, in addition to the main 2-methyladenine zone, a minor zone in the position of 2-methylhypoxanthine. Material eluted from this zone had an absorption maximum in acid solution at 249 mμ., the expected position for 2-methylhypoxanthine (Table 2), which doubtless arises from partial deamination of 2-methyladenine during the acid hydrolysis.

The identification has been neatly confirmed by Ford & Holdsworth (1954), who have shown that when, and only when, synthetic 2-methyladenine or the nucleotide from factor *A* is included in the medium, an *Esch. coli* mutant elaborates mainly factor *A* instead of other B_{12} vitamins.

In a preliminary search, we have failed to find 2-methyladenine in other natural products, namely, the crude purines precipitated by cuprous salts from rumen extract and from fish solubles (kindly prepared by Dr E. S. Holdsworth), crude rumen nucleic acids and yeast nucleic acids. A proportion greater than 1 % of the adenine present would have been detected by the methods used.

A precisely analogous relationship exists between another pair of B_{12} vitamins. We have isolated a few mg. of pure ψ-vitamin B_{12} from pig manure and have confirmed that it yields adenine on acid hydrolysis (Dion *et al.* 1952). It also can be deaminated with hot nitrous acid, yielding a neutral, red, crystallizable and microbiologically active substance, which again appears to be identical with a substance that we have isolated in minute yield from pig manure and call 'Factor *G*'. Deaminated ψ-vitamin B_{12} and factor *G* both yield the expected base, hypoxanthine, on acid hydrolysis (Brown & Lester Smith, 1954). Deamination of ψ-vitamin B_{12} has also been reported by Pfiffner, Calkins & Dion (1954).

Table 1. *Physical and microbiological properties of some B_{12} vitamins*

Factor	Absorption maxima (mμ.)	Electrophoretic behaviour at pH 3	Microbiological potency (in % relative to that of B_{12}): *Esch. coli* mutant† Plate assay	*Esch. coli* mutant† Tube assay	*L. leichmannii* tube assay†	*Ochromonas malhemensis*‡
A	361, 520, 548	Slightly basic	85	30	25	1
ψ-Vitamin B_{12}	—	Less basic than factor *A*	120	20	50	0·2
G (and deaminated ψ-B_{12})	359, 516, 540	Neutral	160	—	20–100*	0·3–3*
H (and deaminated factor *A*)	358·5, 517, 540	Neutral	280	40	15–40*	0·7–2*
F	—	Neutral	130*	50	—	5
I	295, 561, 518, 550	Neutral	125–150*	45–60	35	40–60*

* Non-linear response. † Ford (1953*a*). ‡ Ford (1953*b*).

Table 2. *Properties of purines from factors* A, G *and* H

The exact composition of the two solvent systems is given in the text.

Purine	Absorption maxima (mμ.) pH 2	pH 7	pH 10	R_F values in *n*-butanol plus NH_3	Diethylene glycol + NH_3
Base from factor *A*	266	262·5	270	0·52	0·65
2-Methyladenine	266	262	270	0·53	0·65
Base from deaminated factor *A*	—	—	—	0·27	0·49
Base from factor *H*	249	249·5	261·5	0·25	—
2-Methylhypoxanthine	249·5	249·5	261·5	0·27	0·50
Base from factor *G*	248	—	—	0·15	—
Hypoxanthine	248	248	262·5	0·15	—
Adenine	262·5	259	268	0·38	0·51

The nucleotide can be removed from vitamin B_{12} with little attack on other labile groups by brief treatment with warm concentrated hydrochloric or perchloric acid (Armitage *et al.* 1953). The main cobalt-containing part of the B_{12} molecule so released is a substance still exhibiting microbiological activity towards the *Esch. coli* mutant; it is identical with factor *B* previously isolated from calf faeces and from fermentation liquors (Ford & Porter, 1952). When the treatment with hydrochloric or perchloric acid was applied to ψ-vitamin B_{12}, to factor *A* or to their deamination products, factor *B* was always produced. The latter was identified by microbiological activity, behaviour on paper electrophoresis at various pH values and paper chromatography with *sec.*-butanol. The total hydrolysate from each of the factors was run against a similar B_{12} hydrolysate made with concentrated HCl. Not only were the R_F values of the main (factor *B*) zones identical, but several other zones tallied in the parallel chromatograms: these zones arose from acids produced by partial hydrolysis of labile amide groups (Armitage *et al.* 1953). It is therefore clear that all these B_{12} vitamins are built up by addition of appropriate nucleotides to the one fundamental substance, factor *B*.

SUMMARY

1. By suitable fractionation procedures a number of red cobalt-containing substances other than vitamin B_{12} have been isolated from pig and calf manure. These are factors *A*, *B* and *C* of Ford & Porter (1952) and ψ-vitamin B_{12} of Dion, Calkins & Pfiffner (1952). Others are designated Factors *D*, *E*, *F*, *G*, *H* and *I*.

2. Factor *A* of Ford & Porter (1952) differs from vitamin B_{12} only in having 2-methyladenine in place of 5:6-dimethylbenziminazole.

3. On deamination, factor *A* is converted into a naturally occurring factor *H*, which yields 2-methylhypoxanthine on mild hydrolysis.

4. Similarly, ψ-vitamin B_{12} is deaminated to a naturally occurring hypoxanthine containing factor *G*.

5. ψ-Vitamin B_{12} and factors *A*, *G* and *H* resemble vitamin B_{12} in yielding one fundamental cobalt-containing substance, factor *B*, on removal of their nucleotides with warm concentrated hydrochloric acid.

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The Biosynthesis of Vitamin B_{12}-Like Compounds*

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We have shown (Ford, Holdsworth, Kon & Porter, 1953) that the 'vitamin B_{12}' in extracts of gut contents and faeces is composed in the main of cyanocobalamin and four related compounds, factors *A*, *B* and *C*, and pseudovitamin B_{12}. In general, extracts of animal tissues contain preponderantly cyanocobalamin, whereas in natural materials subjected to bacterial fermentation the other compounds are often present in relatively larger amounts. For example, more than 70% of the 'vitamin B_{12}' in the rumen contents of calves is in the form of factor *A*, whereas the liver contains mainly cobalamin, with sometimes a trace of factor *A* (Ford, Holdsworth & Porter, 1953).

In a recent publication, Gant, Smith & Parker (1954) disclosed that on controlled acid hydrolysis

* Read in part before the Biochemical Society on 16 July 1954 (Ford, Holdsworth & Kon, 1954).

the nucleotide can be removed from cyanocobalamin, factor *A* and pseudovitamin B_{12}. In each instance the 'denucleotided' substance produced was identified as factor *B*. Further tests established that both factor *A* and pseudovitamin B_{12} differ from cyanocobalamin solely in the nature of their nucleotide components.

It is not known what nucleotide, if any, is present in factor *C*. In cyanocobalamin the nucleotide is 5:6-dimethylbenziminazole-α-D-ribofuranose phosphate (Buchanan, Johnson, Mills & Todd, 1950*a*,*b*); the base of pseudovitamin B_{12} has been identified as adenine (Dion, Calkins & Pfiffner, 1952) and that of factor *A* as 2-methyladenine (Brown & Smith, 1954; Dion, Calkins & Pfiffner, 1954). Fig. 1 shows the structural formulae of the three bases. All the compounds are active as cyanocobalamin for *Escherichia coli* 113–3 (Davis), a vitamin B_{12}-requiring mutant strain. Cyanocobalamin, factor *A* and pseudovitamin B_{12} appear to be intrinsically active, and are not necessarily interconverted in the metabolism of the organism. Thus, they can largely be recovered unchanged after passage through the organism. Factor *B*, on the other hand, is normally recovered as factor *C*, sometimes accompanied by traces of the other B_{12} vitamins (Ford, Kon & Porter, 1952). By adding to the growth medium both factor *B* and the nucleotide or the nucleotide base of cyanocobalamin, pseudovitamin B_{12} or factor *A*, we were able to direct *Esch. coli* to the synthesis of the corresponding substances (Ford & Holdsworth, 1954). Thus, with factor *B* and adenine, pseudovitamin B_{12} was produced, in accord with the finding that adenine is present in its nucleotide; and with factor *B* and a number of compounds related to adenine or benziminazole, certain new, 'unnatural' analogues of 'vitamin B_{12}' were produced. We report here the preparation and some properties of these compounds.

EXPERIMENTAL

To each of a series of penicillin culture flasks were added 250 ml. of the vitamin B_{12}-free basal medium of Burkholder (1951), modified by substituting thiomalic for thioglycollic acid. The flasks were plugged with cotton wool and sterilized by autoclaving. To each was then added aseptically 0·5 ml. of a solution of factor *B* (8 μg./ml.) in 30% (v/v) ethanol. To one flask no further addition was made, while to each of the others was added one or another of the different test compounds listed in Table 1, usually in amounts of 5 mg. However, only 1 mg. of cyanocobalamin nucleotide was available for these experiments, and only a few micrograms of factor *A* nucleotide, representing the purified product of the hydrolysis of 0·4 mg. of the factor. The flasks were then each inoculated with 0·2 ml. of a culture of *Esch. coli* 113–3 grown with limiting cyanocobalamin. After incubation for 16 hr. at 30° the cultures were centrifuged. Microbiological test showed that the supernatant liquors contained no vitamin B_{12} activity, and they were discarded. The harvested

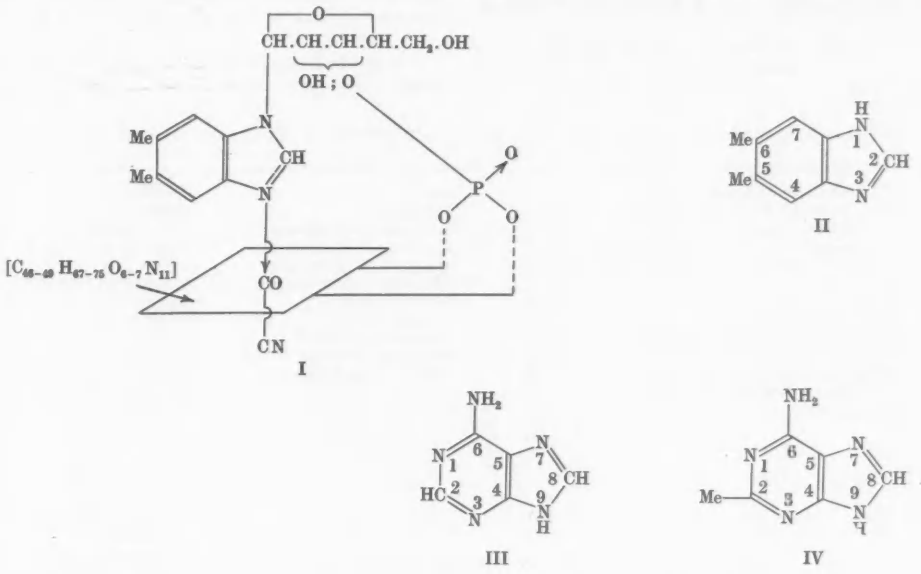

Fig. 1. I, partial formula of cyanocobalamin (Armitage *et al.* 1953); II, 5:6-dimethylbenziminazole; III, adenine; IV, 2-methyladenine.

cells were taken up in 3 ml. 0·033 N-HCl and 0·2 ml. of a 1% solution of NaCN, and heated at 100° for 30 min. The extracts were made to 5 ml. with water and clarified by centrifuging. They were then assayed for 'vitamin B_{12}' with *Esch. coli* 113–3 and with *Ochromonas malhamensis* (see below).

The forms of 'vitamin B_{12}' in the extracts were examined by a bioautographic technique, after paper chromatography and after ionophoresis on paper.

Certain of the experiments were repeated, using radioactive factor *B*, labelled with ^{60}Co. In these instances the extracts were examined by an autoradiographic technique, after chromatography on paper.

Microbiological assay methods

Tests with Esch. coli 113–3. The technique used was that described by Burkholder (1951). The basal medium was modified by substituting thiomalic for thioglycollic acid, and by adding 1 mg. NaCN/100 ml. of 'five-times strength' medium.

Tests with Ochromonas. The technique described by Ford (1953) was used. The 'five-times strength' basal medium was supplemented with 1 mg. NaCN/100 ml.

Other methods

Chromatography. Two solvent systems were used: (*a*) *sec.*-butanol–acetic acid–water–5% (w/v) KCN (100:1:50:0·25, by vol.); (*b*) *sec.*-butanol–aq. NH_3 (sp.gr. 0·88)–water–5% (w/v) KCN (100:1:50:0·25, by vol.). The chromatography was done at 35° on Whatman no. 4 paper. Solutions of cyanocobalamin, pseudovitamin B_{12} and factors *A*, *B* and *C*, each containing 0·2 μg./ml., were used for reference and they and the cell extracts were applied to the paper in 0·005 ml. amounts.

Ionophoresis. The reference standards and cell extracts were examined by ionophoresis on paper with N acetic acid containing 0·01% (w/v) KCN (Holdsworth, 1953).

Bioautographic technique. Esch. coli 113–3 was used as an indicator of vitamin B_{12} activity on the paper chromatograms, in a highly sensitive bioautographic technique described in an earlier publication (Ford & Holdsworth, 1953).

Autoradiographic technique. In the experiments with factor *B* labelled with ^{60}Co, chromatograms of the cell extracts were placed in contact with Ilfex (Ilford, Ltd.) X-ray film for 7 days. The film was then processed as recommended by the manufacturers.

Sources of intermediates. The factor *B* used in these experiments was kindly given to us by Dr Lester Smith of Glaxo Laboratories Ltd., Greenford. It had been prepared by partial hydrolysis of cyanocobalamin (Gant *et al.* 1954) and purified by chromatography and ionophoresis. Dr Lester Smith also provided the nucleotides of cyanocobalamin and factor *A*.

We are indebted to Professor A. R. Todd, F.R.S., for gifts of 2-methyladenine, 2:8-dichloroadenine and 2-methylthioadenine, and to Dr R. E. F. Mathews and Dr J. D. Smith for the 8-azapurines. Certain of the substituted benziminazoles were made by the general procedure outlined by Beaven *et al.* (1949). The remaining substances were available commercially.

RESULTS

In all, forty-eight compounds were tested. Some of them had little or no effect upon the conversion of factor *B* into factor *C*, and these are marked with an asterisk in Table 1. Others modified this process, by 'shunting' the synthesis to one or another of a variety of 'natural' or 'unnatural' B_{12} vitamins. The results obtained with these compounds are set out in Table 2.

It is evident that, when given factor *B* and the nucleotide of factor *A* or of cyanocobalamin, *Esch.*

Table 1. *List of compounds tested for their effect on the biosynthesis of B_{12} vitamins by* Esch. coli

Compounds related to benziminazole	Compounds related to adenine	Various
5:6-Dimethylbenziminazole	Adenine	Guanine*
4:5-Dimethyl-1:2-diaminobenzene	Adenosine*	Guanylic acid*
5-Methylbenziminazole	Adenosine 2′-phosphate*	8-Azaguanine*
3:4-Dimethyl-6-D-ribitylaminobenzene*	Adenosine 3′-phosphate*	Hypoxanthine*
1-Amino-3:4-dimethyl-6-D-ribitylaminobenzene	Adenosine 5′-phosphate*	2-Methylhypoxanthine*
Riboflavin	Adenosine triphosphate*	8-Azahypoxanthine*
Benziminazole	2-Methyladenine	Inosine*
5:6-Dichlorobenziminazole	8-Azaadenine	Xanthine*
4:5-Dichloro-1:2-diaminobenzene	2:6-Diaminopurine	Uracil*
5-Aminobenziminazole	2:8-Dichloroadenine	Uridine*
5-Nitrobenziminazole	2-Methylthioadenine	Cytidine*
4-Chloro-1:2-benztriazole		Thymidine*
Benzthiazole		Thymine*
		5-Methylcytosine*
		Orotic acid*
		Thiamine*
		Nicotinic acid*
		Nicotinamide*
		p-Aminobenzoic acid*
		Folic acid*
		Citrovorum factor*
		Pyridoxamine*
		Riboflavin phosphate*

* These compounds had little or no effect on the conversion of factor *B* into factor *C*.

coli reconstitutes the corresponding vitamin. We had none of the nucleotide of pseudovitamin B_{12} to test, but it seems likely that it would be combined with factor *B* in the same manner. The free nucleotide bases were also effective in 'shunting' the biosynthesis, as is evident from Fig. 2, which illustrates the findings of experiments with factor *B* labelled with ^{60}Co. The autoradiograph shows that, when grown with factor *B* alone, *Esch. coli* converted it into factor *C*, but when adenine was added to the system relatively little factor *C* appeared, and most of the radioactivity was in the pseudovitamin B_{12} position. Adenosine and adenosine 2′-, 3′- and 5′-phosphates were much less effective than the free base in directing the biosynthesis of pseudovitamin B_{12}.

Fig. 3 illustrates again, in diagrammatic form, the effects of certain intermediates in 'directing' the biosynthesis of B_{12} vitamins. It further illustrates the use of ionophoresis on paper, combined with the bioautographic technique, in differentiating between the different forms of 'vitamin B_{12}'.

Table 2. *Biosynthesis of B_{12} vitamins by* Esch. coli 113–3 *when given factor* B *and different compounds structurally related to benziminazole or adenine*

To each flask were added factor *B* (4·0 μg.) and one of the different test compounds (usually 5 mg.) listed in column 1. The flasks were then inoculated with *Esch. coli* as described in the text. The R_F values were obtained with the two solvent systems, the detailed composition of which is given in the Experimental section. The figures for recovery of vitamin B_{12} activity in extracts of cells grown with factor *B* and various test compounds (last two columns) indicate only the relative activities of the different compounds formed for the two micro-organisms. It must be noted that the different B_{12} vitamins have widely different activities for each individual test micro-organism (Ford, 1953). On a weight basis, factor *B* is approximately 20% as active as cyanocobalamin in the *Esch. coli* tube test. It is quite inactive for *Ochromonas*.

Test intermediates in addition to factor *B*	B_{12} vitamins in cell extracts*	R_F		Recovery of vitamin B_{12} activity (expressed as μg. cyanocobalamin) by assay	
		sec-Butanol–acetic acid–water	*sec*-Butanol–NH_3–water	*Esch. coli*	*Ochromonas*
Factor *B* alone (control)	Factor *C*	0·02	0·04	0·16	0·04
+5:6-dimethylbenziminazole	Cyanocobalamin	0·25	0·30	0·97	1·05
+cyanocobalamin nucleotide	Cyanocobalamin	0·25	0·30	0·90	0·82
+5-methylbenziminazole	New factor	0·19	0·28	0·76	0·76
+benziminazole	New factor	0·17	0·24	0·31	0·35
+3:4-dimethyl-6-D-ribityl-aminobenzene	Factor *C*	0·02	0·04	0·13	0·03
+1-amino-3:4-dimethyl-6-D-ribityl-aminobenzene	Factor *C* and cyanocobalamin	0·02 0·25	0·04 0·50	0·50	0·36
+riboflavin	Factor *C* and cyanocobalamin	0·02 0·25	0·04 0·30	0·30	0·18
+5:6-dichlorobenziminazole	New factor	0·35	0·41	0·27	0·46
+4:5-dichloro-1:2-diaminobenzene	New factor	0·35	0·41	0·30	0·50
+4-chloro-1:2-benztriazole	New factor	0·26	0·40	0·19	0·10
+5-aminobenziminazole	New factor	†	0·14	0·22	0·05
+5-nitrobenziminazole	New factor	†	0·29	0·34	0·23
+benzthiazole	Factor *C* and new factor	0·02 0·15	0·04 0·24	0·23	0·15
+adenine	Pseudovitamin B_{12}	0·11	0·085	0·85	0·07
+adenosine	Factor *C* and trace of pseudovitamin B_{12}	0·02 0·11	0·04 0·085	0·20	0·04
+adenosine 2′-phosphate	Factor *C*	0·02	0·04	0·20	0·04
+adenosine 3′-phosphate	Factor *C*	0·02	0·04	0·16	0·04
+adenosine 5′-phosphate	Factor *C*	0·02	0·04	0·25	0·05
+adenosine triphosphate	Factor *C*	0·02	0·04	0·20	0·04
+2-methyladenine	Factor *A*	0·13	0·12	0·28	0·007
+factor *A* nucleotide	Factor *A* and trace of cyanocobalamin	0·13 0·25	0·12 0·30	0·50	0·10
+8-azaadenine	Factor *C* and factor *A* (?)‡	0·02 0·13	0·04 0·12	0·14	0·03
+2:6-diaminopurine	New factor(s)	0·04 (0·06)	0·04 (0·07)	0·12	0·004
+2:8-dichloroadenine	New factor	0·22	0·26	0·17	0·13
+2-methylthioadenine	New factor	0·26	0·17	0·25	0·03

* The compounds listed were sometimes accompanied by other factors in traces.

† No value available.

‡ New spot inseparable from factor *A* by chromatography or ionophoresis. Evidence of incorporation of azaadenine must await isolation of factor, hydrolysis and identification of the base.

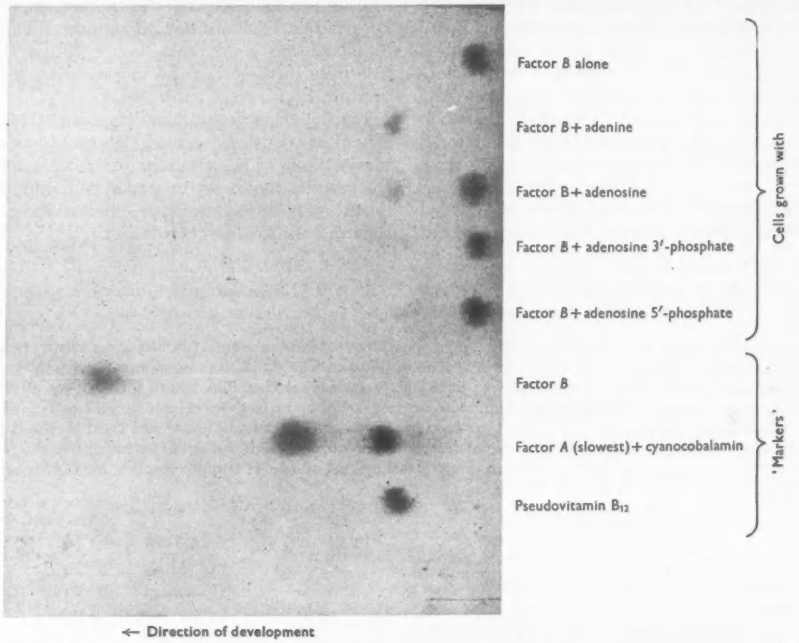

Fig. 2. Autoradiograph of paper chromatogram of factors *A* and *B*, pseudovitamin B_{12} and cyanocobalamin, 'labelled' with ^{60}Co; and of extract of cells of *Esch. coli* grown with 'labelled' factor *B*, and with adenine or certain of its derivatives. Chromatogram developed with *sec.*-butanol–acetic acid–water at 35°. Cells grown in factor *B* alone contain factor *C*.

From the analogues of benziminazole tested, a number of new compounds were produced, some highly active microbiologically for both *Esch. coli* and *Ochromonas*. The latter organism has hitherto been regarded as highly specific in its requirement for cyanocobalamin (Ford, 1953), and it may well prove to display the same pattern of specificity as birds and mammals. We have now undertaken the preparation of certain of the benziminazole derivatives in pure form and on a larger scale, and hope in due course to report the results of growth tests with higher animals. The vitamin B_{12}-like compound formed with benziminazole has already been isolated in pure form. For *Ochromonas* it proved 36% as active as cyanocobalamin, and in *Esch. coli* (tube) assays the compound was 27% as active as cyanocobalamin.

Several other new B_{12} vitamins were produced by adding certain substituted purines to the growth medium (see Table 2).

DISCUSSION

It seems likely that in the mutant strain of *Esch. coli* used in these experiments the requirement for 'vitamin B_{12}' is imposed by a defect in the synthesis of factor *B*. Given this factor and the base of any one of a number of alternative nucleotides, the organism completes the synthesis of the form of 'vitamin B_{12}' containing that particular nucleotide. In contrast, for certain other micro-organisms requiring 'vitamin B_{12}', e.g. *Euglena gracilis*, *Lactobacillus leichmannii* and *Ochromonas malhamensis*, factor *B* is inactive. In the series of compounds tested as intermediates in nucleotide synthesis, two main aspects are of interest: first, the ability of the compound to be incorporated; and secondly its suitability for the formation of substances active for *Ochromonas*.

In the benziminazole series, all the compounds tested gave rise to different forms of 'vitamin B_{12}'. Owing to tautomerism in the symmetrical benziminazole molecule N-1 and N-3 atoms are similar. But with benziminazoles unsymmetrically substituted in the benzene ring, two different nucleotides would presumably be formed by the attachment of the ribotide to the different nitrogen atoms. From factor *B* and 5- (or 6-) methylbenziminazole it seemed that only one new form of 'vitamin B_{12}' was produced. This new substance appeared homogeneous on chromatography and ionophoresis, but

it must be stressed that, if in fact the two monomethyl derivatives were formed they would almost certainly be extremely difficult to separate.

A new form of 'vitamin B_{12}' was produced with the compound 4-chloro-1:2-benztriazole, in which a nitrogen atom replaces the CH of the iminazole ring. This compound, however, was less effective than the benziminazoles in 'shunting' the synthesis.

The appearance of a new form of 'vitamin B_{12}' with benzthiazole was unexpected, since neither the sulphur nor the nitrogen of the thiazole would appear to have a free valency which could combine with ribose to form a nucleotide.

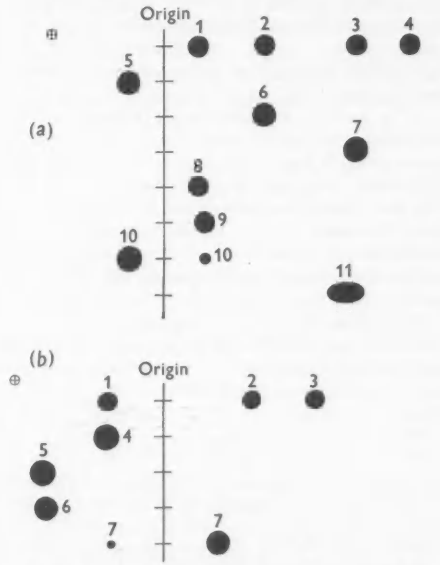

Fig. 3. Ionophoresis on paper of B_{12} vitamins extracted from *Esch. coli* grown with factor *B* and various intermediates. Diagrammatic representation of the bioautographs. (*a*) Ionophoresis at pH 2·2 for 16 hr. at 8v/cm. in N acetic acid containing 0·01% (w/v) KCN. Marker spots: 1, cyanocobalamin; 2, pseudovitamin B_{12}; 3, factor *A*; 4, factor *B*. Spots obtained with extracts of *Esch. coli* grown with factor *B* and different intermediates: 5, factor *B* alone; 6, factor *B* + adenine; 7, factor *B* + 2-methyladenine; 8, factor *B* + 5:6-dimethylbenziminazole; 9, factor *B* + benziminazole; 10, factor *B* + riboflavin; 11, factor *B* + 2:6-diaminopurine. (*b*) Ionophoresis at pH 6·4 for 16 hr. at 8v/cm. in phosphate–citrate buffer containing 0·01% KCN. Marker spots: 1, factor *C*; 2, cyanocobalamin; 3, factor *B*. Spots obtained with extracts of *Esch. coli* grown with factor *B* and different intermediates: 4, factor *B* alone; 5, factor *B* + 5:6-dichlorobenziminazole; 6, factor *B* + 5-nitrobenziminazole; 7, factor *B* + 4-chloro-1:2-benztriazole.

More substances were available in the purine series for testing. Adenine, 2:8-dichloroadenine, 2-methyladenine and 2:6-diaminopurine gave vitamin B_{12}-like substances, but with intermediates having a hydroxyl group in position 6, e.g. hypoxanthine, xanthine, guanine and 2-methylhypoxanthine, there was no significant shift of the synthesis from factor *C*.

None of the adenine nucleotides tested was as effective as the free base in directing the synthesis of pseudovitamin B_{12}. Owing to the unsymmetrical nature of adenine, two different sugar derivatives could be formed by combination at positions 7 and 9 (see Fig. 1). Furthermore, the commonly occurring adenylic acids contain the ribose in a β-linkage, whereas cyanocobalamin nucleotide contains an α-linked sugar molecule. Thus there would seem to be at least four theoretically possible adenine nucleotide derivatives, the 7α- and 7β- and the 9α- and 9β-ribose phosphates. Of these only the 9β compounds have been tested and found not to produce pseudovitamin B_{12}. That the isomerism of the purine–sugar link is important is suggested by the work of Tamm, Folkers, Shunk & Horsfall (1954), who tested the ability of a variety of benziminazole derivatives to inhibit the multiplication of influenza virus. Of all the compounds tested, 5:6-dichloro-1-β-D-ribofuranosylbenziminazole proved especially active, much more so than the dichlorobenziminazole itself. This high activity is very largely conferred by the β-D-ribofuranosyl component of the molecule, and it would be of interest to learn whether the corresponding α-D-riboside is also active.

In view of the specificity of *Ochromonas malhamensis*, which resembles that shown by animals, for 'vitamin B_{12}', it was of particular interest to establish whether any of the new compounds were active or inhibitory for this organism. It appeared that with the loss of methyl groups from the dimethylbenziminazole nucleotide of cyanocobalamin, activity for *Ochromonas* diminished. The compound produced with benziminazole itself had only 36% of the activity of cyanocobalamin. The compound produced with 5-methylbenziminazole has not yet been isolated in pure form and therefore no precise figure is available, but it seems likely that it is somewhat less active than cyanocobalamin.

We were surprised to find that with 5:6-dichlorobenziminazole (or the corresponding diamine) a new factor was produced having the same order of activity as cyanocobalamin for *Esch. coli*, *Lb. leichmannii* and *Ochromonas*. The 5-nitrobenziminazole, but not the 5-aminobenziminazole, led to the production of *Ochromonas* activity. In the purine series, pseudovitamin B_{12}, containing adenine, is inactive for *Ochromonas*, the 2-methyl derivative (factor *A*) has a small but significant

activity and the 'unnatural' analogue from 2:8-dichloroadenine is much more active. The number and variety of compounds so far examined is too small to allow speculation about the kind of nucleotide grouping that confers *Ochromonas* activity, but presumably the active compounds have certain common features either of molecular size or shape or electronic character. It should perhaps be stressed that our work provides no direct proof that these intermediates are actually incorporated into the new factors. The evidence is on the change of activities towards the two test organisms and on the appearance of substances with new chromatographic and ionophoretic properties. But Fantes & O'Callaghan (1954) have shown that when given benziminazole, *Streptomyces griseus* produces a new form of vitamin B_{12} containing this base in its nucleotide.

Such of the B_{12}-group of compounds as are active for *Esch. coli* or *Ochromonas* (with the exception of factor *B* and possibly also factor *C*) can very largely be recovered unchanged after passage through *Esch. coli* or *Ochromonas*. Certain of the 'unnatural' compounds have this property in common with the naturally occurring factors. Thus the compounds obtained from benziminazole and dichlorobenziminazole are intrinsically active and are not first converted into cyanocobalamin.

Our observation that cyanocobalamin was produced when riboflavin was included in the medium gives some support to the view of Woolley (1950, 1951), that the metabolic paths of these two vitamins are closely interconnected. Of course, it need not mean that riboflavin is actually broken down to provide the precursor of cyanocobalamin. It could equally well be that a common precursor of riboflavin and cyanocobalamin nucleotide is normally available in limiting amounts and is used preferentially in the synthesis of riboflavin. Thus the provision of preformed riboflavin might exert a sparing action. Certainly 4:5-dimethyl-1:2-diaminobenzene, which Woolley considers to be the common precursor, is capable of 'shunting' synthesis to cyanocobalamin very effectively.

The finding that riboflavin can promote the synthesis of cyanocobalamin prompts speculation on several reports that it will also stimulate the growth of rats and chicks on vitamin B_{12}-deficient diets (Cooperman, Tabenkin & Drucker, 1952; Hartman, Dryden & Cary, 1951). Certain substituted benziminazoles were also active in the same manner (Emerson *et al.* 1950), and it seems likely that the effect is in each instance due to an enhanced gut synthesis of forms of vitamin B_{12} active for higher animals.

We have found that certain 'wild' strains of *Esch. coli* and *Aerobacter aerogenes* when grown in a medium containing dichlorodiaminobenzene in the amount of 20 μg./ml. synthesize a new form of 'vitamin B_{12}' apparently identical with that formed by the *Esch. coli* mutant from factor *B* and dichlorobenziminazole. In view of this, it is interesting to note that Woolley (1951) found dichlorodiaminobenzene to have relatively little growth inhibitory action on coliform organisms.

SUMMARY

1. In *Escherichia coli* 113–3 the requirement for 'vitamin B_{12}' is probably imposed by a defect in the synthesis of factor *B*, which represents the non-nucleotide portion of the molecule in several of the B_{12} vitamins (Gant *et al.* 1954). Given factor *B*, the micro-organism can be directed to the synthesis of cyanocobalamin, factor *A* or pseudovitamin B_{12} by the provision of the appropriate nucleotide or nucleotide base. For example, with adenine, pseudovitamin B_{12} is synthesized.

2. A variety of compounds related in their chemical structure to adenine or to 5:6-dimethylbenziminazole have been examined for their effect on 'vitamin B_{12}' synthesis in this system. In this way new 'unnatural' analogues of vitamin B_{12} have been obtained. Certain of these new analogues are active for *Ochromonas malhamensis*, an organism previously thought to be specific for cyanocobalamin.

3. Certain other organisms, e.g. wild strains of *Esch. coli* and *Aerobacter aerogenes*, will produce these analogues when the appropriate intermediate is included in the growth medium.

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The Application of the Ceric Sulphate-Arsenious Acid Reaction to the Detection of Thyroxine and Related Substances

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During a study of the urinary excretion products in connexion with the clinical trials of the antithyroxine compound, butyl 4-hydroxy-3:5-diiodobenzoate (Fraser & Maclagan, 1953; Maclagan & Wilkinson, 1951, 1954) it was observed that the urine from patients receiving the drug decolorized the ceric sulphate–arsenious acid reagent of Kolthoff & Sandell (1934). Although a trace of iodide was present, this reagent could not be used for its quantitative estimation because the principal metabolic product, 4-hydroxy-3:5-diiodobenzoic acid, appeared to catalyse the reduction of ceric sulphate by arsenious acid almost as effectively as iodide. Subsequently it was found that many diiodophenols, including thyroxine and diiodotyrosine, gave similar reactions, and it seemed possible that, with the employment of this reagent, a highly sensitive method for the detection of thyroxine and related substances might be developed.

Existing methods for the detection of these compounds, e.g. in paper chromatography, depend either upon the reactions of their amino acid side chains with ninhydrin or diazotized sulphanilic acid, or on autoradiography for ^{131}I-labelled materials only. The former procedure is not sufficiently sensitive to make visible spots containing less than 10 μg. of thyroxine or other iodine-containing amino acids. Moreover, the presence of metabolites such as iodide, which are not amino acids, cannot be demonstrated by this method. The use of the ceric sulphate–arsenious acid reagent would overcome these disadvantages, and the present paper describes the development of such a method, a brief account of which has already appeared (Bowden & Maclagan, 1954).

EXPERIMENTAL

Preparation of reagents. Unless otherwise specified, reagents of the 'AnalaR' grade were used, and all solutions were prepared with 'iodine-free' water (Riggs & Man, 1940). In our experience, water which had been distilled from glass apparatus proved satisfactory without further treatment.

Three primary solutions have been used, the final reagents being made by diluting or mixing these as described below:

(1) Ceric sulphate (10%, w/v) was prepared by adding $Ce(SO_4)_2$, $4H_2O$ (B.D.H. 'low in other rare earths'; 10 g.) to N-H_2SO_4 (100 ml.), previously cooled to 0–5°. The cloudy solution was refrigerated for 1 hr., then clarified by centrifuging or filtration and stored in a refrigerator.

(2) The arsenious acid solution (5%, w/v) was prepared by adding sodium arsenite (Hopkin & Williams's laboratory reagent grade; 5 g.) in small portions to vigorously stirred N-H_2SO_4 (100 ml.) at 0°. If the temperature was allowed to rise or if stirring was omitted, dehydration occurred leading to the precipitation of arsenious oxide. Alternatively, the sodium arsenite (5 g.) was dissolved in N-NaOH (30 ml.) and the solution run dropwise into mechanically stirred 2N-H_2SO_4 (65 ml.) at 0–5°. The volume was then adjusted to 100 ml. with water.

(3) Ceric sulphate (2%, w/v) was prepared by adding $Ce(SO_4)_2$, $4H_2O$ (2 g.) to 4N-H_2SO_4 (100 ml.).

For ordinary paper chromatograms the reagent was prepared by mixing equal volumes of solutions 1 and 2 immediately before use (reagent *A*).

If the paper chromatograms are required for photoelectric scanning, then solutions 1 and 2 were diluted to one-fifth the strength with N-H_2SO_4 before mixing (reagent *B*).

For detection of iodine-containing compounds in aqueous solutions, the same one-fifth strength reagents are used as described. For the detection of iodide in urine, however, solution 3 is used in conjunction with the 1% (w/v) sodium arsenite produced by dilution of solution 2 with N-H_2SO_4.

Aqueous solutions

The method used is a modification of one previously described (Foote, Mackenzie & Maclagan, 1952) for the detection of iodide in urine. Sodium arsenite reagent (1 ml. of 1%) and $Ce(SO_4)_2$, $4H_2O$ reagent (1 ml. of 2%) were added to 1 ml. of the aqueous iodine-containing solution. The time taken to give distinct fading as compared with a blank using 1 ml. of iodine-free distilled water instead of the solution was noted. A standard time of 5 min. was taken as giving minimum detectable quantities at room temperature.

Alternatively, by placing the tubes in a boiling-water bath immediately after mixing, total decolorization in tubes after 4 min. was taken as giving minimum detectable quantities.

It was essential to clean the glass apparatus thoroughly by washing with chromic acid and rinsing with iodine-free distilled water.

Paper chromatograms

The application of the method to paper chromatograms was also studied. One-dimensional chromatograms on Whatman no. 1 filter paper (40 × 10 cm.) were run by vertical ascent for 16 hr. at 20±4° using the butanol–dioxan–ammonia mixture of Gross, Leblond, Franklin & Quastel (1950). The chromatograms were dried in a cabinet fitted with an extraction fan and soda lime traps to remove atmospheric iodine.

Preliminary attempts at spraying the mixed reagent *A* on to the paper were hazardous for the operator and gave very poor results. It appeared that the limiting factor was the time allowed for the reagents to react in the wet state, and after numerous trials the following technique was adopted.

The mixed ceric sulphate–arsenious acid reagent *A* (*ca.* 10 ml.) was applied evenly to a sheet of chromatographic paper of the same size as the original chromatogram by laying it flat on a sloping, clean glass plate and allowing the reagent to flow down the paper so that it was evenly wetted and free from wrinkles and air bubbles. The glass plate was then placed on a flat surface and the wetted paper covered by the dry chromatogram. A second glass sheet was placed on top and firmly pressed down, so that the chromatogram was also evenly wetted. The papers were allowed to remain in this position for varying periods (usually 30 min.) according to the sensitivity required. The plates were then separated and the papers dried in an iodine-free atmosphere. The positions of iodine-containing compounds were indicated by white spots on a yellow background. Each chromatogram thus produced two similar records.

Permanent records. The dry chromatograms faded after a few days as the result of action of atmospheric iodine, and the strongly acid nature of the reagent caused the paper to disintegrate within a few weeks, hence it was necessary to devise some means of preparing more permanent records. For many purposes, simply tracing the spots on a second sheet of paper was adequate, but photographic methods were much to be preferred, as they provide a permanent record of the relative intensity of the spots. Direct photography using an Ilford tricolour blue filter no. 304 and paper gave a record of white spots on a black background. It was simpler and quicker to back the chromatogram with Ilford reflex document paper no. 50 and expose for a few seconds to ultraviolet light from an Osram black glass MBW/V lamp. The paper stained by ceric sulphate blocked the transmission of ultraviolet light, whilst the white spots did not. Thus the record consisted of black spots on a white background.

Semi-quantitative measurements. A scanning device based upon that of Nanninger & Bink (1951) was employed so that rough quantitative measurements of the amounts of iodine compounds present could be determined. The dry chromatogram was interposed between a source of ultraviolet light (Osram MBW/V lamp) and a Spekker fluorimeter photoelectric cell connected to a Cambridge reflecting galvanometer (sensitivity 2200 mm./μA at 2 m.). The photoelectric cell was mounted in a block of wood on which was placed a series of wooden sheets each bored with a hole, the diameter of which was equal to that of the cell. These wooden sheets enabled the length of the light path to be altered so that convenient deflexions of the galvanometer could be obtained. The spots on the chromatograms were placed in position on the top of these blocks and covered with a thin Perspex sheet bearing an outline of the cell to facilitate alinement of the spots being measured, the light source and the cell. The apparatus is illustrated in Fig. 1.

Chromatograms for use in this apparatus were treated with the one-fifth strength ceric sulphate–arsenious acid reagent (reagent *B*). This increases the sensitivity of the estimation at low concentrations without seriously altering the blank reading. It was important to standardize rigidly the amount of reagent used and also the development and running times for this method. The chromatograms had to be completely dry—this was achieved in 30 min. at 20°/1 mm. in a vacuum oven.

Determination of iodine liberated by treatment with ceric sulphate from iodine-containing compounds

The sensitivity of the reaction for a number of organic iodine compounds was studied, and an attempt was made to correlate the results with the rate of liberation of iodine from the compounds when treated with ceric sulphate under standard conditions as follows.

The iodine-containing compound (100 mg.) was dissolved or finely suspended in *tert.*-butanol (2 ml.) and vigorously shaken mechanically at 22±2° for 40 min. with 10%, w/v, ceric sulphate solution in N-H_2SO_4 (10 ml.). The liberated

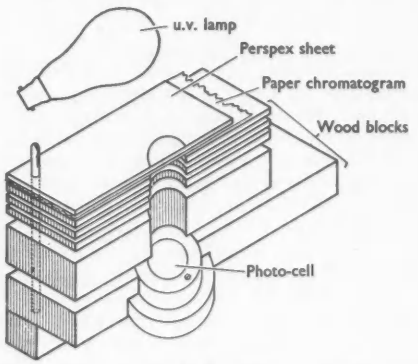


Fig. 1. Apparatus for scanning paper chromatograms (half-section).

iodine was then extracted into benzene (20 ml.) by shaking for a further 5 min. The benzene layer was washed 3 times with water (10 ml., 5 ml., 5 ml.) to remove H_2SO_4 and extracted with 0·1 N-$Na_2S_2O_3$ (10 ml.). The benzene residue was washed twice with water (5 ml.) and the washings were added to the $Na_2S_2O_3$ extract, which was then back-titrated with 0·1 N-I_2. The results obtained with a number of substances are discussed below.

The nature of the oxidation products of the iodophenols has been subjected to preliminary investigation, but we are not yet able to define their structure. We are, however, indebted to Dr I. D. P. Wootton of the Postgraduate Medical School of London for examining the infrared spectrum of one of the products. This has shown that aromatic character was lost on oxidation.

RESULTS AND DISCUSSION

Aqueous solutions

Table 1 shows the minimum concentrations of potassium iodide, thyroxine, triiodothyronine and diiodotyrosine in aqueous solution required for fading and decolorization under the conditions described above. The reaction is more sensitive at 100° than at room temperature, and very small amounts can be detected.

However, for detection of iodide in urine the reagents need slight modification. The 2 % $Ce(SO_4)_2$, $4H_2O$ is made up in 4 N-H_2SO_4 instead of N-H_2SO_4. The increase in acid is necessary to prevent the precipitation of ceric phosphate from some urines. This method is similar to that of Foote *et al.* (1952) but is preferred, as the reagents are more stable and slightly more sensitive, giving a minimum detectable concentration of 40–60 μg. I^-/100 ml. at room temperature.

Paper chromatograms

Still smaller amounts of iodine-containing compounds can be detected on paper chromatograms. The minimum concentration detectable by spraying was 3·0 μg. potassium iodide, whereas the plate technique was about 300 times more sensitive (limit, 0·01 μg.).

Kolthoff & Sandell (1934) have pointed out that the effect of iodides on the reduction of the ceric ion by arsenite is catalytic. It would appear that when the reagent is sprayed on the chromatogram it dries too rapidly for the effect of small traces of iodide to become visible, whilst in the alternative procedure sufficient time is allowed for the catalytic effect to be obvious.

Typical results are shown in Table 2. Fig. 2 shows the range within which the semi-quantitative method may be used. For thyroxine, in the range 0·1–0·4 μg. the correlation between concentration and galvanometer reading is linear with some scatter. The upper limit is fixed by the diameter of the photoelectric cell. Since the amount of light reaching the cell depends on the area of the spot, spots must not be overloaded with material. Thus it was found necessary to dilute stronger solutions so that the 10 μl. applied to the paper contained about 0·3 μg. thyroxine or 0·09 μg. potassium iodide.

Mechanism of the reaction

Some information has been obtained concerning the nature of the iodine compounds which respond to the reagent. In preliminary experiments it was found that the presence of a free phenolic hydroxyl or similar group was essential, for no derivatives of iodophenols in which the hydroxyl group was protected by methylation or esterification gave the reaction in solution. Similar results were obtained on paper. When present in stoicheiometric amounts,

Table 1. *Detection of iodine compounds in aqueous solution*

Compound	Minimum concentration causing fading at room temperature (μg./ml.)	Minimum concentration causing decolorization at 100° in 4 min. (μg./ml.)
Potassium iodide	0·2	0·02
Diiodotyrosine	0·4	0·08
Thyroxine	2	0·1
Triiodothyronine	2	0·2

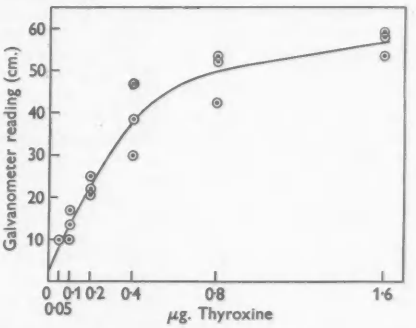


Fig. 2. Photoelectric scanning of paper chromatogram by transmitted ultraviolet light.

Table 2. *Detection of iodine compounds on paper chromatograms*

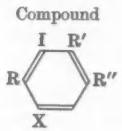
Compound	Smallest amount detectable by: Visual or direct photography. u.v. photoelectric measurement (μg.)	Smallest amount detectable by: u.v. photography or u.v. visual (μg.)
Potassium iodide	0·01	0·04
Diiodotyrosine	0·02	0·06
Thyroxine	0·1	0·3
Triiodothyronine	0·1	0·3

several phenols which did not contain iodine also decolorized the reagent, but this effect was not catalytic and was easily distinguished from the behaviour of iodides and iodophenols. The catalytic effect was also shown by 4-amino-3:5-diiodobenzoic acid, so it would appear that the presence of an electron-donating substituent is necessary.

In order to examine the possibility that the effects observed with iodophenols were due to liberation of iodine, various iodophenols or their derivatives were treated with excess ceric sulphate under standard conditions and the liberated iodine was titrated with sodium thiosulphate. The results (Table 3) show that those compounds (nos. 1–15) which liberated any significant quantity of iodine had a free hydroxyl group in a position *ortho* to an iodine atom. Conversely, those substances (nos. 16–24) which did not possess this structural feature released little or no iodine on treatment with ceric sulphate, with the single exception of 3:5-diiodothyronine (no. 16). Since 3:5-diiodotyrosine (no. 8) gives up its iodine more readily than most diiodophenols, it would seem that the alanine side chain may be partly responsible.

From the figures in the last column of Table 3, it is clear that there is a correlation between the smallest amounts of a number of compounds detectable on paper chromatograms and the percentage of iodine set free by ceric sulphate. Qualitatively, the iodo compounds fall into two groups: those which readily release iodine and give the reaction on paper, and those which are resistant to oxidation to iodine and do not give the reaction on paper. The conclusion may therefore be drawn that the detection of iodophenols by the ceric sulphate–arsenious acid reagent is due to the ease of liberation of iodine and not to any specific property of the molecule as a whole. The only exception to this conclusion which

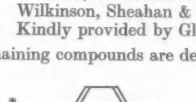
Table 3. *Iodine liberated from derivatives of iodophenols by treatment with excess* 10 % *(w/v) ceric sulphate at* 20° *for* 40 *min.*

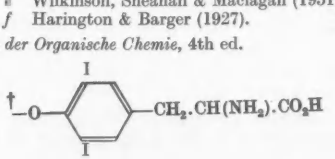


No.	R	R′	R″	X	Ref.	Iodine liberated: mg./100 mg. compound	Iodine liberated: Percentage of total iodine	Smallest amount of compound detectable on paper chromatograms (μg.)
1	HO	H	CH_3	I	—	29·9	42·4	0·01
2	HO	H	COOH	I	—	7·2	11·0	0·02
3	HO	H	$COOCH_3$	I	—	11·4	18·1	0·02
4	HO	H	$COOC_4H_9$-*n*	I	*a*	6·4	11·2	0·01
5	HO	H	CHO	I	—	8·9	13·1	0·05
6	HO	H	COC_2H_5	I	*b*	23·5	37·2	0·02
7	HO	H	NO_2	I	—	13·9	21·7	0·02
8	HO	H	$CH_2.CH(NH_2).COOH$	I	—	26·6	45·3	0·02
9	HO	H	†	I	*c*	5·2	7·9	0·1
10	HO	H	†	H	*c*	6·4	10·9	0·1
11	HO	H	COOH	H	—	6·3	13·1	0·1
12	HO	H	$COOC_4H_9$-*n*	H	*d*	4·4	11·1	0·2
13	HO	H	CHO	CH_3O	—	10·2	22·3	0·02
14	H	HO	$COOC_4H_9$-*n*	I	*a*	2·5	4·4	0·05
15	H	HO	CHO	I	—	15·2	22·4	0·02
16	*	H	$CH_2.CH(NH_2).COOH$	I	*c*	5·7	11·8	0·02
17	CH_3O	H	COOH	I	—	0	0	>10·0
18	CH_3O	H	$COOCH_3$	I	—	0	0	>10·0
19	CH_3O	H	CN	I	*e*	0	0	>10·0
20	$CH_3.COO$	H	$COOCH_3$	I	*e*	0·6	1·1	0·5
21	$CH_3.COO$	H	CHO	I	*b*	0	0	5·0
22	$C_2H_5O.COO$	H	CHO	I	*b*	0·6	1·1	5·0
23	*	H	CN	I	*f*	0	0	2·0
24	H	CH_3O	CHO	I	*b*	0	0	>10·0
			Potassium iodide	—	—	71·2	93·0	0·01
			Potassium iodate	—	—	0	0	0·03

a Sheahan, Wilkinson & Maclagan (1951).
b Wilkinson, Sheahan & Maclagan (1951*a*).
c Kindly provided by Glaxo Laboratories Ltd.
d Maclagan, Sprott & Wilkinson (1952).
e Wilkinson, Sheahan & Maclagan (1951*b*).
f Harington & Barger (1927).

The remaining compounds are described in Beilstein's *Handbuch der Organische Chemie*, 4th ed.





we have investigated is the case of potassium iodate, which reacts with the ceric sulphate–arsenious acid mixture, but does not yield any iodine with ceric sulphate alone (Table 3). It has been shown by adding arsenious acid before ceric sulphate, that this anomaly is due to preliminary reduction of potassium iodate by the arsenious acid.

The method as described is relatively insensitive to aliphatic iodine-containing compounds such as iodoacetic acid, but we are informed by Dr I. R. Bush (St Mary's Hospital, London) that spots of such compounds are detectable if the chromatograms are previously treated with alkali. This causes hydrolysis of the compound with the liberation of iodide. Since alkaline solutions of thyroxine contain iodide after prolonged storage, we tried the effect of alkali on chromatograms bearing spots of this substance, but were unable to demonstrate any increased sensitivity.

SUMMARY

1. Methods are described for the detection of inorganic and organic iodine-containing compounds in solution and on paper chromatograms using ceric sulphate and arsenious acid.

2. In solution 0·2 μg. potassium iodide and 2·0 μg. thyroxine are detectable at room temperature. On paper chromatograms the method is sensitive to 0·01 μg. potassium iodide and 0·1 μg. thyroxine.

3. Methods for making permanent records of the chromatograms are described. The general procedure can be adapted for the semi-quantitative estimation of thyroxine in the range 0·1–0·4 μg.

4. The mechanism of the reaction has been studied and the conclusion reached that the method depends upon the liberation of iodine from the compounds by oxidation with ceric sulphate. With one exception (potassium iodate), only those compounds which readily yield iodine when treated with ceric sulphate can be detected on paper with this reagent.

5. The reaction is given by all the aromatic compounds studied which contain one or more iodine atoms in positions *ortho* to an electron-donating substituent such as the hydroxyl or amino group.

The work was aided by grants from the Medical Research Council and from the Governors' Discretionary Fund of Westminster Hospital to whom grateful acknowledgement is made. Thanks are also due to Miss Shena Anderson for skilled technical assistance, and to Glaxo Laboratories Ltd. for gifts of thyroxine and other compounds.

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The Inhibition of Horse-Liver Esterase by Rhodamine B

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(*Received* 29 *April* 1954)

Horse-liver esterase is inhibited by low concentrations of several fluorescent dyes (Burch, 1954). It was considered that this inhibition might be of special interest for the theory of enzyme action, since the combination of small fluorescent molecules with proteins may be studied by an independent physical method: that of the polarization of fluorescence, introduced by Weber (1952*a*, *b*), and applied by Laurence (1952) to the study of the reversible adsorption of dyes by serum albumin. Combination between enzyme and substrate or inhibitor is fundamental to the accepted mechanisms of enzyme action and its reversible inhibition; but, except in the important case of the haem

* Present address.

enzymes, evidence for rapidly reversible combination is provided by kinetic data only. Extra-kinetic evidence for such combination, and its quantitative role in enzyme action and inhibition, would be very desirable for the hydrolytic enzymes in which no active centres have yet been identified.

Rapid-flow techniques would be needed to observe the combination of enzyme with fluorescent substrate; inhibitors may, however, be studied by ordinary methods, and the theory of competitive inhibition is very closely linked with the theory of normal enzyme action. Preliminary experiments indicated that the inhibition of horse-liver esterase by rhodamine B is competitive in type; and it was hoped to obtain a quantitative comparison between dissociation constants determined kinetically and by fluorescence studies, and to find the number of combination sites per unit weight of protein in the enzyme preparation.

It will be shown, however, that on further study the inhibition proved to be kinetically much more complex than the usual competitive pattern. Combination between dye and enzyme has been demonstrated by the fluorescence method, but the quantitative connexion between the results of the two types of investigation has not been established. A possible mechanism for the inhibition is put forward.

THEORY OF THE FLUORESCENCE METHOD

The polarization of the fluorescent light from molecules in solution depends on the relation between the lifetime of the excited state and the rotational relaxation time. Rhodamine B in aqueous solution gives a very low polarization p_F: in solvents of higher viscosity the relaxation time is increased and the polarization rises to a limiting value p_F^0; in glycerol this value is attained. In aqueous solution, combination of the dye with protein increases the relaxation time, and the polarization of fluorescence is raised to a value p_B (Weber, 1952*a*). The proportion of dye in the free and bound forms, in any solution, may be calculated from the polarization p of the fluorescence of the solution if p_B, p_F and R, the ratio of the fluorescent intensity of the bound dye to that of the free, have been determined. If the molar concentrations of bound and free dye are C_B and C_F respectively, and we put $3p/(3+p)=p'$, Weber's (1952*a*) eqn. 8 shows that

$$C_F/C_B = R(p'_B - p')/(p' - p'_F), \qquad (1)$$

p'_F may be measured directly, and p'_B found as the limiting value of p' when sufficient enzyme is present to bind all the dye.

To obtain R, comparison is made between the intensities of fluorescence of two identical cells, containing dye in equal concentrations. Cell x contains dye free in solution, and cell y contains enzyme in addition, so that the dye is almost wholly bound. Let the observed intensity ratio, (cell y)/(cell x), be R_1. To obtain the true relation between the fluorescent intensities of the molecular species present in the two cells, two corrections are required. (i) A difference between the absorption spectra of the two solutions will lead to a difference in the proportions of both exciting and emitted light which are absorbed. It may readily be shown that R_2, the intensity ratio corrected for this effect, is given by

$$R_2 = R_1 a_y A_y (1-e^{-a_x l})(1-e^{-A_x L}) / a_x A_x (1-e^{-a_y l})(1-e^{-A_y L}), \qquad (2)$$

where the suffixes refer to solutions x and y, and a = absorption coefficient at the wavelength of the exciting light; A = absorption coefficient for the observed emitted light; l = cell depth in the direction of the exciting beam; L = cell depth in the direction of observation of the emitted light. When al and AL are small, $R_2 = R_1$. (ii) The proportion of the total fluorescent intensity which is observed depends also on the polarization: Weber's (1952*a*) analysis shows that if

$$R_2 = R_3(1+p_y/3)/(1+p_x/3), \qquad (3)$$

R_3 represents the true ratio between the fluorescent intensities of solutions y and x. If cell y contains bound dye only, $R = R_3$, but in practice this may not be possible, since the high enzyme concentrations necessary may introduce such large absorption coefficients that the calculation of R_3 from R_1 (eqn. 2) becomes inaccurate. In this case, a simple proportion argument, together with eqn. 1, shows that

$$R = R_3/\{1-(R_3-1)(p'_B-p'_y)/(p'_y-p'_F)\}, \qquad (4)$$

and R is determined by measurements of intensities, absorption coefficients and polarizations. Then in any solution C_F/C_B may be found from eqn. 1 by measurement of p'.

For a simple equilibrium combination between dye and enzyme, $(e-C_B)\,C_F/C_B = K$, where e is the total molar concentration of binding centres present, and K is the dissociation constant. Hence, if $e = c[e_a]$, where $[e_a]$ is the enzyme concentration in arbitrary units and c is a constant,

$$[e_a]\,C_F/C_B = K/c + C_F/c. \qquad (5)$$

This equation may be used to find K and C from the experimental observations.

EXPERIMENTAL

Enzyme. The purified horse-liver esterase preparation previously described (Burch, 1954) was used throughout.

Rhodamine B. A commercial sample of the dye was recrystallized 4 times: twice as the hydrated base, which separated slowly in green plates, at room temperature, after addition of dilute NaOH to aqueous solutions of the hydrochloride (Noelting & Dziewonsky, 1905); and twice as the hydrochloride from very small volumes of hot water. The hydrochloride was finally dried *in vacuo* over H_2SO_4. Stock solutions used for kinetic and polarization work were stored in dark bottles.

Dolinsky (1949) reports extinction coefficients for pure rhodamine B hydrochloride in dil. NH_3 solutions. $E^{1\,\mathrm{mg./l.}}_{1\,\mathrm{cm.}}$ is given as $0{\cdot}233 \pm 0{\cdot}001$ at the wavelength of the maximum (553 ± 2 mμ.); and E_{520}/E_{553} as $0{\cdot}36 \pm 0{\cdot}01$. The present sample in the specified solvent, examined in a Beckman spectrophotometer gave a maximum extinction at 554–555 mμ., where $E^{1\,\mathrm{mg./l.}}_{1\,\mathrm{cm.}}$ was 0·234; E_{520}/E_{554} was 0·35. Chromatography in water on alumina columns showed that the commercial dye contained a component which remained stationary at the top of the column. In the purified sample this could not be detected, and the coloured material travelled as a single band.

Anhydrous rhodamine B base, for the freezing-point measurements, was prepared from the purified hydrochloride by crystallization of the hydrated base in the previous manner. The washed crystals were dried to constant weight *in vacuo* over P_2O_5.

Depressions of the freezing point were determined in the usual way (Findlay, 1941) using a Beckman thermometer. Successive additions of the anhydrous base were made to benzene of a grade supplied commercially for this purpose.

Inhibition kinetics. The rates of ester hydrolysis were measured by the continuous potentiometric titration method, using the apparatus and detailed procedures previously described (Burch, 1954). Unless otherwise indicated, initial reaction velocities were used and reaction mixtures were made up containing substrate, phosphate buffer, KCl, rhodamine B hydrochloride and nearly one equivalent of NaOH/g. mol. dye. Titration to the standard pH of 7·4 was completed as usual after equilibration in the reaction vessel, and the reaction was started by addition of 0·5 ml. enzyme solution to the 50 ml. of mixture. The accuracy of measurement of each reaction rate depended on the individual conditions of the run, and was estimated as described previously (Burch, 1954). All error limits given are estimated in this way, unless otherwise stated.

Fluorescence studies. Solutions, unless otherwise indicated, contained 0·01 M phosphate buffer (0·008 M-Na_2HPO_4–0·002 M-KH_2PO_4, pH 7·4) and 0·1 M-KCl. Reagents had been previously boiled to remove CO_2. A solution of the enzyme preparation, dialysed against 0·01 M phosphate, pH 7·4, and containing 1660 esterase units and 3·56 mg. N/ml. was freeze-dried and stored in the cold. Samples of the powder were dissolved in 0·1 M-KCl to the original volume, and very small amounts of insoluble matter removed by high-speed centrifuging. These stock solutions showed an esterase activity identical with that before freeze-drying. In the fluorescence work, enzyme concentrations were expressed in arbitrary units, as fractions of the concentration of these stock solutions, and denoted by $[e_a]$.

In polarization and intensity measurements, the Hg line at 546 mμ. was used for excitation. Light from a mercury discharge was filtered through a cell of $NaNO_2$ solution, a didymium filter Corning no. 5120, and a green filter Corning no. 401. The emitted light was observed through a red filter complementary to the exciting light. No emission could be detected from the enzyme preparation alone. Solutions were contained in closed optical cells, of depth 1 cm. in the direction of the exciting beam, and thickness 2 cm. in the direction of observed emission. Polarizations were measured by the method of Weber (1952*a*), using his calibrated apparatus at 25°. Fluorescent intensity comparisons between two cells were made visually at room temperature, using a fluorimeter with a divided incident beam and calibrated adjustable diaphragms in the observation pathways. Unpolarized exciting light was used in all measurements except those of p_p at high dye concentrations, when a Nicol prism was placed in the incident beam. This increased the measured polarization value, permitting more accurate observations on solutions of very low polarization; but it seriously diminished the intensity of illumination, and was therefore only useful at high dye concentrations. The relation $p = P/(2 - P)$ (Perrin, 1929), where P and p are the polarizations observed with polarized and natural exciting light respectively, was used to calculate p when P was measured.

RESULTS

Kinetic characteristics of the inhibition

Inhibitor and substrate concentrations. Fig. 1 shows the results of four experiments, each at a constant concentration of enzyme and of methyl butyrate. The rhodamine concentration was varied, and the reciprocal of the hydrolysis rate, $1/v$, plotted against the dye concentration [I]. In each case the velocity decreased steadily as [I] rose; and, at any fixed value of [I], the percentage inhibition became greater as the substrate concentration fell, indicating a competitive type of inhibition. In normal competitive inhibition, eqn. 11 of Lineweaver & Burk (1934) shows that the apparent value of the Michaelis constant, K_A, is linear with [I], and at constant substrate concentration $1/v$ is linear with [I]; but in Fig. 1 each experiment gave a curve concave downwards.

Fig. 2 shows the results of a series of experiments at constant enzyme concentration. To allow for small differences in the active enzyme concentrations used on different days, a velocity determination with no inhibitor was made each day, and all measured velocities were multiplied by a factor referring them to a standard enzyme concentration which gave a limiting velocity V of 25·0 μM/min. The resulting values of $1/v$ were plotted against 1/[S], and each series of points at a given dye concentration lay reasonably closely about a straight line of intercept $1/V = 0{\cdot}04$ min. μM^{-1}. Thus the inhibition was truly competitive with respect to substrate: the presence of inhibitor altered only the apparent value of the Michaelis

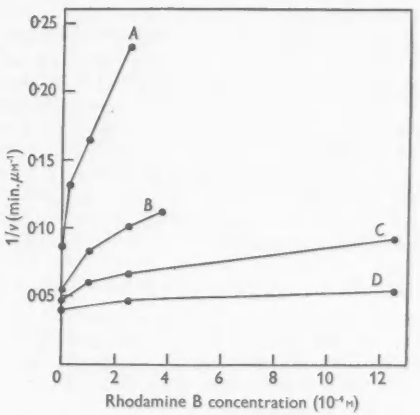

Fig. 1. Inhibition: effect of rhodamine at various concentrations of substrate. Phosphate concn. $1{\cdot}0 \times 10^{-3}$ M. Methyl butyrate concn. (M × 10^{-3}): *A*, 2·27; *B*, 5·68; *C*, 14·2; *D*, 56·8.

constant, leaving the maximum velocity unchanged. But the influence of [I] was not that of simple competitive inhibition; in Fig. 3 the K_A values obtained from the gradients of the straight lines of Fig. 2 are plotted against the rhodamine concentrations, and the result is a continuous curve rather than a straight line.

Reversibility. Three types of evidence showed that the inhibition was rapidly established and reversible, so that the experimental results referred to an equilibrium condition.

(i) The reaction mixtures were made up containing inhibitor but no substrate. After equilibration and titration to the standard pH, either the substrate was added and followed immediately by enzyme, or enzyme was added first and allowed to remain with the inhibitor for varying periods before the addition of 2 ml. of substrate solution started the reaction. There was no detectable acid liberation or consumption when enzyme and inhibitor were together without substrate. Table 1 shows the results of two experiments. Some decrease in reaction rate resulted from leaving enzyme with inhibitor before substrate addition; but this decrease was no greater than that caused by leaving the enzyme under the same conditions (of pH, temperature, and concentrations of KCl, phosphate and enzyme) with no inhibitor present (Burch, 1954). In both cases the loss in activity was progressive, and was about 10 % per 20 min., so that the percentage inhibition was unaffected.

(ii) The enzyme was added as usual to a mixture of inhibitor and substrate. After the reaction had proceeded for 10 min., the mixture was suddenly diluted by the addition of an equal volume of a solution containing substrate, KCl and phosphate. The inhibitor concentration was thereby halved, while the total amount of enzyme, and the concentrations of all other components, were unchanged. The reaction rate immediately increased, becoming equal to that in a control experiment at the lower dye concentration. The progress curves are shown in Fig. 4. The solutions added at the dilution points were previously warmed to 25°, and brought approximately to the standard pH by addition of the quantity of alkali judged necessary. This quantity was slightly underestimated, as shown by the discontinuities in the curves; but the pH of the reaction mixture fell by less than 0·15 unit during the dilution of each run. The reaction rates obtained from the dilution of each run. The reaction rates obtained from the gradients of the curves are given in the

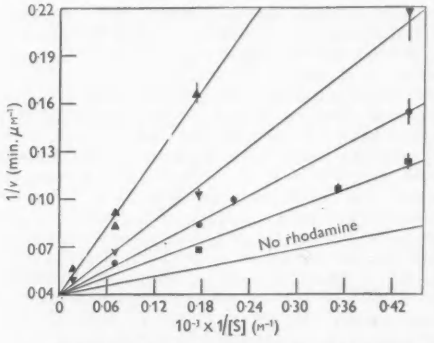

Fig. 2. Variation of hydrolysis rate with methyl butyrate concentration at various concentrations of inhibitor. Phosphate concn. $1{\cdot}0 \times 10^{-3}$ M. Rhodamine B concn. (M $\times 10^{-4}$): ■—■, 0·25; ●—●, 1·0; ▼—▼, 2·5; ▲—▲, 12·5. Estimated error limits shown as vertical lines through the points. Results with no inhibitor taken from Burch (1954).

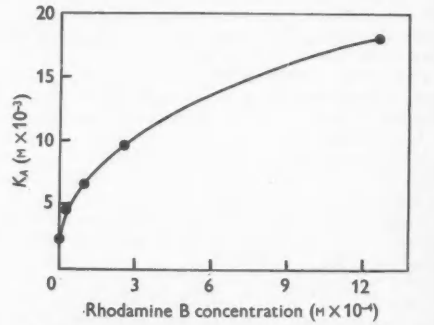

Fig. 3. Effect of rhodamine concentration on the apparent value of the Michaelis constant for methyl butyrate. Results from Fig. 2.

Table 1. *Speed of the inhibitory action of rhodamine B on the esterase*

Mixtures containing $1{\cdot}0 \times 10^{-3}$ M phosphate, 0·1 M-KCl, and $1{\cdot}0 \times 10^{-4}$ M rhodamine B were equilibrated in the reaction vessel and titrated to pH 7·4. Either enzyme (E) or methyl butyrate solution (S) was introduced; and after the time interval shown, the last component was added and the reaction rate measured by the continuous potentiometric titration method described in the text, p. 99. Final substrate concn. $= 4{\cdot}55 \times 10^{-3}$ M. Two separate experiments are shown.

Experiment	1	1	2	2	2
1st addition	S	E	S	E	E
Time interval (min.)	2	19½	3	23½	41
2nd addition	E	S	E	S	S
Reaction rate (μM/min.)	9·6	8·95	9·45	8·2	7·85

(Estimated error in all reaction rates, ±0·3 μM/min.).

figure. On dilution the rate of *A* (Fig. 4) rose to 1·10 × its initial value, and became equal (within less than 1 %) to the rate in the corresponding period of reaction *B*. Larger increases in the rate of *A* could not be obtained in dilution experiments, because of the insensitivity of the rates to changes in dye concentration (Fig. 3) and because the maximum and minimum volumes which could be used in the reaction vessel prevented dilutions greater than twofold.

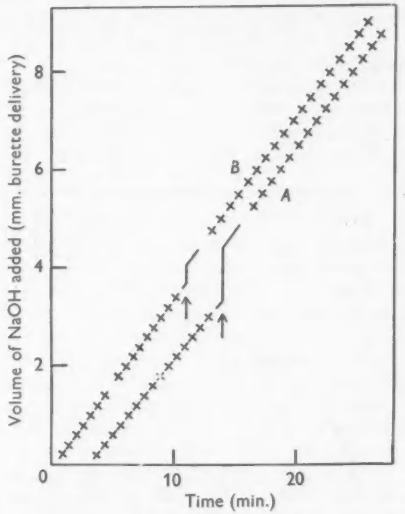

Fig. 4. Dilution experiment showing reversibility of inhibition. × ×, experimental null-points in the continuous potentiometric titration of reaction mixtures. Initial solutions, volume 40 ml., contained 0·1 M-KCl, $0{\cdot}667 \times 10^{-3}$ M phosphate, $14{\cdot}2 \times 10^{-3}$ M methyl butyrate, and $12{\cdot}5 \times 10^{-4}$ M rhodamine (reaction *A*) or $6{\cdot}25 \times 10^{-4}$ M rhodamine (reaction *B*). After equilibration, enzyme was added and the reactions followed. At ↑ each mixture was diluted by the addition of 40 ml. solution containing 0·1 M-KCl, $0{\cdot}667 \times 10^{-3}$ M phosphate, $14{\cdot}2 \times 10^{-3}$ M methyl butyrate, and no rhodamine (reaction *A*) or $6{\cdot}25 \times 10^{-4}$ M rhodamine (reaction *B*). Reaction rates (mm. burette divisions/min.) were: *A*, initially 0·305, after dilution 0·337; *B*, initially 0·346, after dilution 0·335. 1 mm. micrometer burette division = 0·0359 ml. of 0·0696 M-NaOH.

(iii) Evidence for the speed with which the equilibrium degree of inhibition was established was obtained from the linearity of the progress curves of ester hydrolysis. If the inhibitor acted slowly on the enzyme, a progressive decrease in reaction rate with time would be expected when enzyme was added to a mixture of inhibitor and substrate; while if the inhibitor was only slowly displaced from the enzyme by the substrate, a progressive increase in reaction rate would have occurred when substrate was added to a mixture of enzyme and inhibitor. The progress curves of the reactions of Table 1 showed no such difference in form depending on the order of addition of components, and the progress curves of the reactions of Figs. 2 and 4 were substantially linear for periods of about 10 min.

Enzyme concentration. In normal competitive inhibition the percentage inhibition at constant values of [S] and [I] is independent of enzyme concentration. The observed dependence of K_A on [I] (Fig. 3), in which K_A is approximately linear with the square root of [I], could result if an equilibrium of the form $2E + I \rightleftharpoons E_2I$ was responsible for the inhibition. But in this case the percentage inhibition at constant [S] and [I] would necessarily increase with rising enzyme concentration. The results of an experiment testing this point are given in Table 2. The reactions were carried out at room temperature, using a Marconi glass electrode pH meter to record null points at pH 7·40 during the continuous titration. Reaction mixture (25 ml.), in an open vessel, contained substrate and inhibitor. After titration to pH 7·40, different volumes of an enzyme solution were added to start the reaction. Thereafter additions were made from a 2 ml. burette, at intervals of about 90 sec., of a sodium hydroxide solution whose concentration was varied according to the amount of enzyme used. The phosphate concentration was varied similarly, so that each addition of alkali gave a pH increment of about 0·18 unit in every run. The reaction velocity was proportional to enzyme concentration over a tenfold range, both with and without inhibitor. The percentage inhibition was thus independent of enzyme concentration.

Phosphate concentration. In the absence of inhibitor, the rate of hydrolysis of methyl butyrate by the enzyme is independent of phosphate

Table 2. *Influence of enzyme concentration on reaction rates with and without inhibitor*

All reaction mixtures contained 0·1 M-KCl and 14×10^{-3} M methylbutyrate. Rates were measured at room temperature by a continuous titration procedure using an ordinary pH meter: for details see text, this page.

	No rhodamine		$12{\cdot}4 \times 10^{-4}$ M rhodamine			
Phosphate (10^{-3} M)	1·07	10·7	0·53	1·07	4·27	6·40
Enzyme (ml.)	0·3	3·0	0·3	0·44	1·5	3·0
Reaction rate (μM/min.)	41·4	406	29	42	144	273
Rate/ml. enzyme	138	135	97	95	96	91

concentration. This has been shown both at high and low substrate concentrations (Burch, 1954). The inhibition by rhodamine B was also found to be independent of phosphate concentration from $0{\cdot}5 \times 10^{-3}$M to $2{\cdot}0 \times 10^{-3}$M.

Inhibition of ethyl butyrate hydrolysis. When ethyl butyrate was used as substrate instead of the methyl ester, the inhibition by rhodamine B showed the same characteristics, in that it was competitive with respect to substrate, and the graph of K_A against [I] was concave towards the [I] axis. In normal competitive inhibition K_A/K_m is independent of the substrate used, being a function of inhibitor concentration only. This is also true for certain other possible inhibition mechanisms based on the competitive pattern. However, Table 3 shows that K_A/K_m was greater with ethyl butyrate than with the methyl ester, at equal dye concentrations.

Inhibition with 50×10^{-4}M rhodamine. Segal, Kackmar & Boyer (1952) have postulated a type of inhibition in which the inhibitor combines with the enzyme at a point different from the substrate-binding centre, and the resulting enzyme–inhibitor compound has enzymic activity with kinetic constants different from those of the uninhibited enzyme. If the result of the combination with inhibitor is a reduction in the affinity of the enzyme for the substrate, with no change in the rate of breakdown of the enzyme–substrate complex, the inhibition is termed 'apparent competitive', since the apparent value of the Michaelis constant is altered, while the maximum velocity is unchanged. It differs from normal competitive inhibition in giving a graph of K_A against [I] which is concave towards the [I] axis, obeying the equation

$$K_A = K_m(1 + [I]/K_1)/(1 + [I]/K_1').$$

(This may be seen from eqn. 6 below, when K_1 and K_2 are very large so that the truly competitive component of the inhibition is absent.)
Thus, putting $f = [I]/(K_A/K_m - 1)$,

$$f = K_1K_1'/(K_1' - K_1) + K_1[I]/(K_1' - K_1)$$

and K_A increases from K_m, at $[I] = 0$, towards the asymptotic value K_mK_1'/K_1, when $[I] \gg K_1'$; while f increases linearly with [I].

In order to show whether this mechanism accounted for the inhibition results, the data of Fig. 3 were replotted in Fig. 5 as f against [I]. The result appeared to be a curve concave downwards, indicating that as [I] increased, K_A continued to rise steadily without approaching an asymptotic value. Estimation of the accuracy of the K_A determinations is difficult: in this figure the vertical line through each point represents a deviation of $\pm 10\%$ in the K_A value. It was considered that a definite conclusion as to the form of this graph required another point at a higher value of [I]. K_A for methyl butyrate hydrolysis was therefore determined in the presence of 50×10^{-4}M rhodamine, by the method

Table 3. *Comparison of the effects of rhodamine B on the hydrolysis of two separate substrates*

The inhibition was in each case competitive with respect to substrate: K_A denotes the apparent value of the Michaelis constant in the presence of inhibitor.

	Methyl butyrate		Ethyl butyrate	
Rhodamine (10^{-4}M)	K_A (mM)	K_A/K_m	K_A (mM)	K_A/K_m
0	2·32	—	0·62	—
1·0	6·5	2·8	2·5	4·0
12·5	17·9	7·7	9·9	16·0

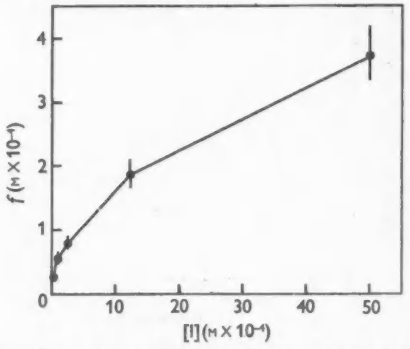

Fig. 5. Effect of rhodamine concentration on the inhibition of methyl butyrate hydrolysis. Results from Fig. 3. $f = [I]/(K_A/K_m - 1)$. Vertical lines show deviations of $\pm 10\%$ in K_A values.

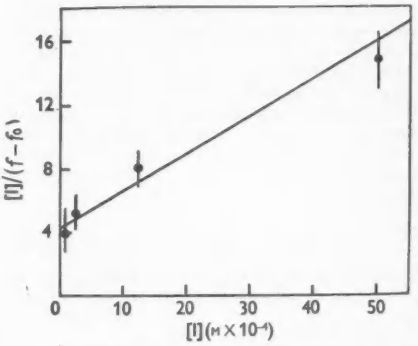

Fig. 6. Effect of rhodamine concentration on the inhibition of methyl butyrate hydrolysis. Results from Fig. 5. f_0 = value of f when $[I] \to 0 = 0{\cdot}3 \times 10^{-4}$M. Vertical lines show deviations of $\pm 10\%$ in K_A values.

used in the experiments of Table 2, except that the reaction volume was 30 ml., and the temperature was maintained at 25°. This simple technique was adequate, since the high value of K_A allowed high substrate concentrations and reaction rates to be employed. On including this point in Fig. 5 it became clear that the graph was curved, so that the results did not conform with 'apparent competitive' inhibition.

A more complex inhibition mechanism is discussed below: 'apparent competitive' and true competitive inhibitions are assumed to be acting simultaneously, and it is shown that in this case $[I]/(f-f_0)$ should be linear with [I], where f_0 is the value of f when $[I] \to 0$. In Fig. 6 f_0 is taken as $0{\cdot}30 \times 10^{-4}$ M, and the inhibition results of Fig. 5 are plotted in this form. As before, vertical lines through the points represent deviations of $\pm 10\%$ in the K_A values.

Properties of rhodamine B in solution

The possibility presented itself that rhodamine B might reversibly form a dimer in aqueous solution. If the monomeric form inhibited competitively while the dimer was inert towards the enzyme, the increase in K_A would be nearly proportional to the square root of the total dye concentration. Ghosh & Sengupta (1938) have postulated such association for rhodamine B in dilute aqueous solution, but alternative explanations of their experimental results are available (Wawilow, 1942), and in all these studies solutions of the hydrochloride of rhodamine B were used. No data are available on the relevant properties of the uncharged base, which is the form present at pH 7·4. Experiments were therefore carried out to investigate the behaviour of the dye in solution under conditions closely comparable with those of the inhibition work.

Partition coefficients. The distribution of rhodamine B between benzene and an aqueous phase of pH 7·4, containing 0·1 M-KCl and 4×10^{-3} M phosphate at 25°, was examined. Dye concentrations in the separated layers were determined colorimetrically: in benzene, rhodamine B is colourless, but the addition of an equal volume of ethanol was found to give deeply coloured solutions. The partition coefficient (concn. in benzene/concn. in aqueous layer) was found to have a constant value of $8{\cdot}5 \times 10^2$ over a 40000-fold range of dye concentrations, from 7×10^{-9} M to 3×10^{-4} M in the aqueous phase. Thus the rhodamine could only dimerize in the aqueous medium if it also did so in the benzene layer.

Depression of the freezing point. Rhodamine B base in benzene was found to give depressions of the freezing point in agreement with those calculated for monomeric dye, at concentrations up to 0·082 M. At 0·147 M the depression was 91 % of the calculated value: since this solution contained over 7 % of solute, a slight deviation from ideal behaviour is not unexpected. All the observed depressions were much greater than those calculated assuming a degree of dimerization in the aqueous medium sufficient to account for the inhibition data of Fig. 3.

Effect of benzene on the inhibition. It remained possible that the results of the distribution experiments did not truly represent the relation between the benzene solutions of rhodamine, used for freezing-point measurements, and the aqueous solutions of the kinetic work, since in the partition experiments each phase was necessarily saturated with the other. At 25° the benzene layer thus contained about 0·03 g. mol. water/l. and since this represents less than one molecule of water per dye molecule (at the upper dye concentrations used), it is very improbable that it fundamentally altered the state of aggregation of rhodamine in benzene. But the solubility of benzene in water is rather greater, and the dye concentrations in this phase were much lower so that a change in the molecular state of the dye due to the presence of benzene was possible. In this case the inhibitory action of the dye on liver esterase should have been changed markedly by the addition of benzene to the reaction mixture. Table 4 shows the results of experiments in which the effect of benzene on the esterase reaction rates was tested, by the method used to investigate the inhibition at 50×10^{-4} M rhodamine. In the reactions with benzene present, the mixtures contained water previously saturated with benzene and carefully

Table 4. *Effect of benzene on the enzymic hydrolysis of methyl butyrate, in the presence and absence of rhodamine B*

Reaction mixtures contained 0·1 M-KCl. Benzene was added as a saturated solution in water, its final concn. being approximately half the saturation value. Hydrolysis rates were measured at 25° by a continuous titration procedure using an ordinary pH meter: for details see text, this page.

Methyl butyrate (mM)		17·15	17·15	17·15	2·34	2·34
Phosphate (mM)		3·3	3·3	5·6	0·67	0·67
Rhodamine B (mM)		1·25	1·25	0	0	0
Enzyme (ml.)		1·0	1·0	1·0	0·25	0·25
Reaction rate (μM/min.)	no benzene (v_1)	152	153	211	29·1	31·7
	benzene present (v_2)	158	160	199	34·2	35·7
v_2/v_1		1·04	1·05	0·94	1·17	1·13

Table 5. *Polarization of fluorescence of rhodamine B bound to esterase*

Solutions contained 0·01 M phosphate buffer pH 7·4 (0·008 M-Na_2HPO_4–0·002 M-KH_2PO_4) and 0·1 M-KCl, at 25°. High enzyme concentrations and low dye concentrations were used, in order to determine p_B. For method of measuring polarizations see text, p. 99.

Enzyme concn. $[e_a]$*	0·1	0·1	0·1	0·375	0·375	0·375
Rhodamine ($M \times 10^{-7}$)	2·0	4·0	6·0	2·0	4·0	6·0
p	0·262	0·255	0·248	0·270	0·270	0·270
$p' = 3p/(3+p)$	0·241	0·235	0·229	0·248	0·248	0·248

* Arbitrary units.

Table 6. *Polarization of fluorescence of free rhodamine B*

Solutions contained 0·01 M phosphate buffer pH 7·4 (0·008 M-Na_2HPO_4–0·002 M-KH_2PO_4) and 0·1 M-KCl, at 25°. For method of measuring polarizations see text. P = polarization of fluorescence with polarized exciting light; p = polarization of fluorescence with natural exciting light.

Rhodamine concn. ($M \times 10^{-7}$)	5	50	100	200	400
P	—	—	0·07	0·06	<0·035
$\frac{P}{2-P} = p$	0·045	0·045	0·036	0·031	<0·018
$\frac{p}{1+\frac{1}{3}p} = p'$	0·045	0·045	0·036	0·031	<0·018

freed from liquid droplets. The final benzene concentration was about half the saturation value. Benzene had small effects on the reaction rates, both with rhodamine present and with no inhibitor at high and low substrate concentrations. The kinetic constants were thus altered somewhat by the change in medium, but the very large effect, which would be expected if the dye associated in the purely aqueous solution but not in the presence of benzene, was not observed.

Fluorescence studies

Polarization experiments. Preliminary observations showed that successive additions of enzyme to a solution of rhodamine B increased the polarization steadily towards a limiting value; and at a fixed enzyme concentration the polarization fell as the dye concentration increased. A series of experiments was carried out, in each of which the enzyme concentration was constant, while successive additions of a concentrated rhodamine B solution were made from an 'Agla' micrometer syringe. The polarization results were shown as follows to relate to a reversible equilibrium state: at the end of an experiment the mixture was diluted with a solution of dye in buffer, to reduce the enzyme concentration only. The resulting lower polarization value was always equal to that of a solution of the same final composition freshly prepared with no intermediate dilution step.

Polarizations at high enzyme and low dye concentrations (Table 5) indicated that $p_B = 0{\cdot}270$, $p'_B = 0{\cdot}248$. p_F was determined at a series of concentrations as shown in Table 6. At the higher concentrations polarized exciting light was used so that P_F was measured and p_F calculated. At concentrations below 5×10^{-6} M, p_F was constant at 0·045; and at higher concentrations depolarization occurred and p_F fell. A solution of rhodamine B in glycerol gave a polarization p^0_F, of 0·270. Thus p_B was equal to p^0_F, indicating that the dye was bound by molecules or particles too large to permit an estimate of their size from the value of p_B.

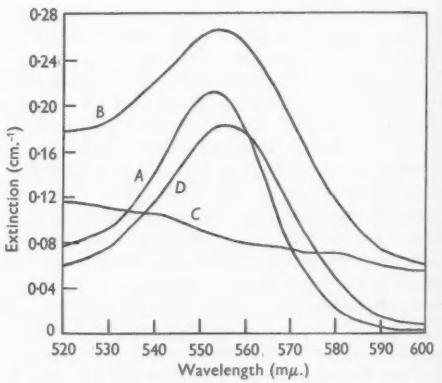

Fig. 7. Absorption spectra (determined in the Beckman spectrophotometer) of the following solutions:

Solution	*A*	*B*	*C*
Rhodamine B concn. ($M \times 10^{-6}$)	1·99	1·99	0
Enzyme concn. $[e_a]$	0	0·374	0·374

All solutions contained 0·1 M-KCl and 0·01 M phosphate buffer, pH 7·4. Curve *D* is the difference between curves *B* and *C*, and represents bound rhodamine. Polarization p' of solution $B = 0{\cdot}234$. Enzyme concn. $[e_a]$ in arbitrary units.

Table 7. *Effect of concentration on the fluorescent intensity of free rhodamine B*

The fluorescent intensity of each solution, relative to that of an arbitrary standard solution of rhodamine, was measured as described in the text. All solutions contained 0·01 M phosphate buffer pH 7·4 (0·008 M-Na_2HPO_4–0·002 M-KH_2PO_4) and 0·1 M-KCl. Absorption coefficients a and A, for exciting and observed emitted wavelengths respectively, were determined in a Beckman spectrophotometer and used to calculate the correction terms for light absorption in each solution. l = Cell depth in the direction of the exciting beam = 1 cm.; L = cell depth in the direction of observation of the fluorescent light = 2 cm.

Rhodamine ($M \times 10^{-7}$)	3·99	5·99	9·95	13·9	19·8	48·8
Observed relative intensity	0·891	1·43	2·11	2·90	4·14	7·07
$\frac{1-e^{-al}}{al}$ (correction factor for absorption of exciting light)	0·955	0·94	0·90	0·865	0·812	0·62
$\frac{1-e^{-AL}}{AL}$ (correction factor for absorption of observed emitted light)	0·99	0·985	0·975	0·965	0·955	0·90
Observed relative intensity divided by $\frac{1-e^{-al}}{al} \times \frac{1-e^{-AL}}{AL} \times$ concn.	0·24	0·26	0·24	0·25	0·27	0·26

Absorption spectrum of bound dye. Fig. 7 shows the absorption spectrum of a solution of dye and enzyme, in which the dye was almost all bound, as shown by the polarization. The spectrum of the enzyme alone in the same concentration is shown, and the difference between the curves gives the spectrum of the bound dye. In relation to that of the free dye, it was shifted towards the red, and its maximum was lower.

Fluorescent intensities. (i) *Free dye.* Intensity comparisons were made between each of a series of rhodamine B solutions and an arbitrary standard solution. The observed relative intensities were corrected for absorption, as in eqn. 2, and Table 7 shows that they were then proportional to concentration, between 4×10^{-7} M and $4{\cdot}9 \times 10^{-6}$ M. Higher concentrations were not used because of the uncertainty in the large absorption corrections involved.

(ii) *Bound dye.* Table 8 shows two experiments to find R (see the theoretical section). In Expt. 2, the absorption of fluorescent light by the enzyme was too great for accurate determination of the absorption correction: maximum and minimum probable values are given. The maximum figure gave a value for R agreeing with the more reliable Expt. 1.

Displacement by substrate. Both competitive and 'apparent competitive' inhibitors should be displaced from combination with the enzyme by sufficiently high concentrations of substrate. In Table 9, comparison of solutions 2 and 4 shows the large fall in polarization which was caused by the presence of ethyl acetate. The fall in pH, due to substrate hydrolysis by the enzyme, was minimized by using this slowly hydrolysed substrate, a temperature of 20°, and concentrated buffer. At the end of the experiment the pH of solution 4, quickly measured with a glass electrode, was found

Table 8. *Ratio of fluorescent intensities, bound/free rhodamine*

The fluorescent intensity of solution y was compared with that of solution x by the method described in the text. All solutions contained 0·01 M phosphate buffer pH 7·4 (0·008 M-Na_2HPO_4–0·002 M-KH_2PO_4) and 0·1 M-KCl. R, the ratio of the fluorescent intensity of the bound dye to that of the free, was then calculated by the steps explained in the theoretical section. The necessary measurements of absorption coefficients were made in a Beckman spectrophotometer; and the polarization of solution y was determined as described in the text, p. 99.

Experiment	1	2
Solution y		
Rhodamine B concn., $M \times 10^{-7}$	6·0	19·9
Enzyme concn., $[e_0]$	0·10	0·374
Solution x		
Rhodamine B concn., $M \times 10^{-7}$	6·0	19·9
Observed intensity ratio, R_1	1·77	1·48
R_2 (from eqn. 2)	1·92	1·73–1·97
p_y	0·248	0·254
R_3 (from eqn. 3)	2·05	1·85–2·11
p'_y	0·229	0·234
R (from eqn. 4)	2·30	1·98–2·30

Table 9. *Displacement of dye by substrate*

The polarization of fluorescence of each solution, at 20°, was determined as described in the text, p. 99.

Soln. no.	Soln. composition	Polarization (p')
1	0·05 M phosphate, pH 7·4, 0·1 M-KCl, 5×10^{-7} M rhodamine B	0·045
2	4 ml. solution (1) + 0·1 ml. stock enzyme solution	0·187
3	4 ml. solution (1) + 0·2 ml. ethyl acetate	0·045
4	Solution (3) + 0·1 ml. stock enzyme solution	0·055
5	Solution (2) + 0·06 ml. N-HCl	0·176

to be 6·8. Hydrochloric acid was added to solution 2, bringing its pH to 6·7: little or no change in polarization resulted (solution 5).

The effect of ethyl acetate was not due to inactivation of the enzyme. Immediately after the experiment, the esterase activity of solution 4 was measured, by adding 0·04 ml. of it to 10 ml. of the standard reaction mixture of the drop-titration test (Burch, 1954) containing ethyl butyrate and bromthymol blue. The resulting reaction rate showed the enzyme to be fully active. The concentrations of rhodamine, ethyl acetate and hydrolysis products introduced were too small to interfere with this activity test.

Comparison of the fluorescent intensities of solutions 1 and 3 showed that the presence of ethyl acetate increased the intensity of fluorescence of the free dye by a factor of 1·3, with no change in absorption spectrum. By this intensity change alone, the presence of the ester would have caused a fall in the polarization of fluorescence of the mixture of free and bound dye; but the following calculation shows that this effect was small compared with the observed difference between solutions 2 and 4. By eqn. 1, at constant C_F/C_B, the reduction in R would have raised the term $(p'_B-p')/(p'-p'_F)$ by a factor of 1·3. Taking p' initially as 0·18 it would thereby have become 0·17. Since the observed final value was only 0·055, C_F/C_B cannot have remained constant: that is, displacement of the rhodamine by the ethyl acetate must have been nearly complete. The only alternative explanation of the polarization change would be an almost complete quenching of the fluorescence of the bound dye by the ester; but such an extreme difference between its effects on the bound and the free rhodamine seems very unlikely.

Equilibrium between dye and enzyme. Using the measured values $p'_B=0·248$, $p'_F=0·045$, and $R=2·30$ in eqn. 1, C_F and C_B were calculated for each mixture of enzyme and dye in the polarization experiments. At dye concentrations above 10^{-5} M, values for p'_F were obtained from Table 6 by interpolation. The resulting values of $[e_a]\,C_F/C_B$ were plotted against C_F; Fig. 8 shows the results at low dye concentrations and Fig. 9 those at high; and the same straight line is drawn in both figures.

DISCUSSION

Inhibition kinetics

The assumption that rhodamine B inhibits liver esterase by interaction with the enzyme, rather than with the substrate, is justified by the low molar concentrations of inhibitor used, compared with the concentrations of substrate. It has been assumed that the concentration of dye free in solution was not appreciably reduced by the presence of the enzyme, so that the quantity [I] in all equations was equal to the total concentration of rhodamine added. The number of enzyme active centres present was almost certainly much too small to bind an appreciable fraction of the dye, by any ordinary inhibition

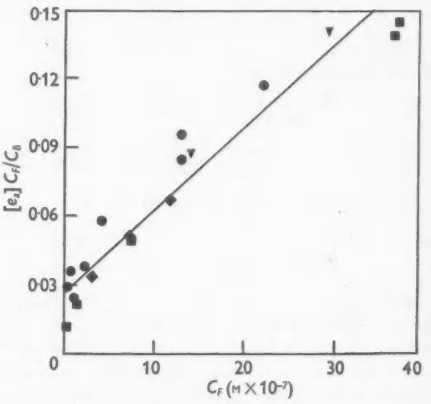

Fig. 8. The equilibrium between rhodamine B and esterase, as shown by the polarization of fluorescence. Enzyme concn. $[e_a]$: ●, 0·0125; ◆, 0·0175; ■, 0·05; ▼, 0·10. C_F = concn. of free dye, C_B = concn. of bound dye. C_F and C_B in each solution were calculated from the measured polarization of fluorescence, as described in the text. All solutions contained 0·1 M-KCl and 0·01 M phosphate buffer pH 7·4 (0·008 M-Na_2HPO_4–0·002 M-KH_2PO_4) at 25°. For method of measuring polarizations, see text, p. 99. ——, straight line identical with that of Fig. 9.

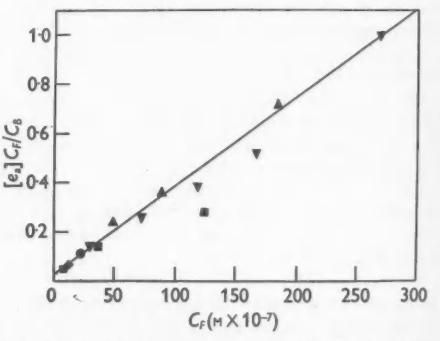

Fig. 9. The equilibrium between rhodamine B and esterase, as shown by the polarization of fluorescence. Enzyme concn. $[e_a]$: ●, 0·0125; ◆, 0·0175; ■, 0·05; ▼, 0·10; ▲, 0·25. C_F = concn. of free dye, C_B = concn. of bound dye. C_F and C_B in each solution were calculated from the measured polarization of fluorescence, as described in the text, p. 98. All solutions contained 0·1 M-KCl and 0·01 M phosphate buffer pH 7·4 (0·008 M-Na_2HPO_4–0·002 M-KH_2PO_4) at 25°. For method of measuring polarizations see text. ——, straight line identical with that of Fig. 8.

mechanism. Dye concentrations were not less than $2{\cdot}5 \times 10^{-5}$ M, and active centre concentrations of this order would have entailed a turnover number for each centre of the order of 0·8 min.$^{-1}$ (for ethyl butyrate hydrolysis at 20°). This value seems unreasonably low. Moreover, Boursnell & Webb (1949) have inhibited this esterase preparation with di*iso*propyl phosphorofluoridate containing ^{32}P and found 1 g. atom of phosphorus to be bound per 1×10^5 g. protein. If this is taken to indicate the number of esterase centres present, the concentration of active centres in the inhibition experiments with rhodamine B was only about 5×10^{-9} M.

Binding of the dye by adsorption at less specific sites on the protein is also possible, but two types of evidence show that it is very unlikely to have reduced the free-dye concentration appreciably. The percentage inhibition, at constant rhodamine and substrate concentrations, was independent of enzyme concentration, instead of falling when more enzyme was added; and extrapolation of the measurements of the polarization of fluorescence shows clearly that, at the low enzyme and high dye concentrations used in the kinetic work, the polarization was equal to that of the free dye, suggesting that a negligible fraction was bound to protein.

The inhibition has been shown to be rapidly established and reversible, to be competitive with respect to substrate, but to yield graphs of K_A against [I] which can be explained neither by the normal competitive mechanism, nor by the 'apparent competitive' inhibition of Segal *et al.* (1952). Inhibition mechanisms involving a complex of the type E_2I are eliminated by the proportionality of the inhibited reaction rate to enzyme concentration, while association of the dye in solution is ruled out by measurements of freezing-point depressions and partition coefficients, together with the small effect of benzene on the reaction rates.

The possibility that the esterase preparation may consist of a mixture of enzymes must be considered, since its activity has not definitely been shown to be due to a single type of active centre (Burch, 1954). A mixture of two independent types of centre, acting on a single substrate, gives a graph of $1/v$ against $1/[S]$ which is concave downwards; and the curvature depends on the square of the difference between the K_m values of the two types of centre (Burch, 1954). The activity of the present enzyme preparation could thus be due to several esterases if their Michaelis constants were effectively equal, since straight-line graphs were obtained both with methyl butyrate and with the ethyl ester (Burch, 1954). Inhibition by rhodamine B does not affect the observed maximum velocity, therefore it can act only on the apparent values of the individual Michaelis constants. If these are changed in the same ratio, and thus remain equal at all values of [I], the system behaves as a single enzyme, and a mechanism still remains to be found to account for the results. If there is any quantitative difference between the actions of the inhibitor on the separate components of the system, the two K_A values will differ and the graphs of $1/v$ against $1/[S]$ in the presence of inhibitor must then be concave downwards rather than straight. Since the observed graphs (Fig. 2) were not significantly curved, it is clear that the existence of more than one type of active centre in the preparation cannot in itself account for the form of the K_A–[I] relationship; and the straight lines of Fig. 2 are evidence that the esterase activity of the preparation is uniform.

An inhibition mechanism which accounts for the observed kinetics will now be proposed. It is assumed that the inhibitor combines with the enzyme at two separate points. When it combines at the substrate-binding centre, as in normal competitive inhibition, the resulting complex, EI, is enzymically inactive. The inhibitor may also combine at a different point, and this complex, EI′, has enzymic activity with changed kinetic constants. Its affinity for the substrate is reduced, while the rate of breakdown of the complex EIS is equal to that of ES. It may also be completely inhibited by further combination of inhibitor at the substrate-binding centre. Thus competitive and 'apparent competitive' inhibitions are acting simultaneously; and the hypothesis may be formulated as follows:

$$\begin{aligned}
&\mathrm{E} + \mathrm{S} \rightleftharpoons \mathrm{ES} \xrightarrow{k_3} \mathrm{E} + \text{products}\\
&\mathrm{E} + \mathrm{I} \rightleftharpoons \mathrm{EI}\\
&\mathrm{E} + \mathrm{I} \rightleftharpoons \mathrm{EI}'\\
&\mathrm{EI}' + \mathrm{S} \rightleftharpoons \mathrm{EIS} \xrightarrow{k_3'} \mathrm{EI}' + \text{products}\\
&\mathrm{EI}' + \mathrm{I} \rightleftharpoons \mathrm{EI}_2\\
&k_3' = k_3
\end{aligned}$$

As pointed out by Segal *et al.* (1952), the generalized stationary state treatment of such a reaction scheme leads to very complex expressions; a special case in which each individual equilibrium is maintained, however, gives simpler results. The graphs of $1/v$ against $1/[S]$ at constant [I] are linear only in this special case, so that the straight lines of Fig. 2 justify the equilibrium treatment for the present system. We may therefore put:

$$[\mathrm{E}]\,[\mathrm{S}]/[\mathrm{ES}] = K_m, \qquad [\mathrm{E}]\,[\mathrm{I}]/[\mathrm{EI}] = K_i,$$
$$[\mathrm{E}]\,[\mathrm{I}]/[\mathrm{EI}'] = K_1, \quad [\mathrm{EI}']\,[\mathrm{S}]/[\mathrm{EIS}] = K_m',$$
$$[\mathrm{EI}']\,[\mathrm{I}]/[\mathrm{EI}_2] = K_2,$$

and it follows that

$$[\mathrm{ES}]\,[\mathrm{I}]/[\mathrm{EIS}] = K_1 K_m'/K_m$$

which may be termed K_1'. Manipulation of these equations in the ordinary way, assuming

$$v = k_3[\mathrm{ES}] + k_3'[\mathrm{EIS}],$$

shows that v is related to [S] by the ordinary Michaelis expression; that if $k_3' = k_3$ the maximum velocity is unchanged by the presence of inhibitor, and that

$$K_A = K_m \frac{1 + [\mathrm{I}]/K_1 + [\mathrm{I}]/K_i + [\mathrm{I}]^2/K_1K_2}{1 + [\mathrm{I}]/K_1'}. \quad (6)$$

Putting $f = \dfrac{[\mathrm{I}]}{K_A/K_m - 1}$, eqn. 6 gives

$$f = \frac{1 + [\mathrm{I}]/K_1'}{1/K_i + 1/K_1 - 1/K_1' + [\mathrm{I}]/K_1K_2}, \quad (7)$$

and when $[\mathrm{I}] \to 0, f \to f_0$, where

$$f_0 = \frac{1}{1/K_i + 1/K_1 - 1/K_1'}. \quad (8)$$

Combination of eqns. 7 and 8 gives

$$\frac{[\mathrm{I}]}{f - f_0} = \frac{K_1K_2/f_0}{K_1K_2/K_1' - f_0} + \frac{[\mathrm{I}]}{K_1K_2/K_1' - f_0}. \quad (9)$$

The graph of $[\mathrm{I}]/(f - f_0)$ against [I] is thus linear, and its slope and intercept, together with the value of f_0, determine the several equilibrium constants. The straight line of Fig. 6 yields the following values, for the inhibition of methyl butyrate hydrolysis:

$$K_1K_2 = 5{\cdot}3 \times 10^{-8}\ \mathrm{M}^2,$$
$$K_1' = 1{\cdot}2 \times 10^{-4}\ \mathrm{M},$$
$$1/(1/K_1 + 1/K_i) = 0{\cdot}24 \times 10^{-4}\ \mathrm{M}.$$

Since K_i, K_1, and K_2 refer to reactions between enzyme and inhibitor only, they must be independent of the substrate used. But K_1' may differ for different substrates, giving a change in the value of K_A/K_m at a given value of [I], as found in Table 3. For ethyl butyrate hydrolysis in the presence of $12{\cdot}5 \times 10^{-4}$ M rhodamine, K_A/K_m was found to be 16·0. Hence K_1' for this substrate may be calculated (from eqn. 6) to be $3{\cdot}0 \times 10^{-4}$ M. In $1{\cdot}0 \times 10^{-4}$ M rhodamine, K_A/K_m should then be 4·04, which compares well with the observed value of 4·0. This agreement provides independent evidence for the proposed inhibition mechanism, since the constants derived from the inhibition of methyl butyrate hydrolysis account for the relation between [I] and K_A/K_m for the ethyl ester.

It must be emphasized that this hypothesis of the mechanism of inhibition can only be regarded as tentative; in common with all reaction mechanisms based on kinetic data, it can only be shown to fit the observations, and cannot be demonstrated as a unique solution to the problem. Furthermore, it involves three independent constants whose values can only be obtained experimentally. Simpler mechanisms, such as the ordinary competitive or the 'apparent competitive' modes of inhibition, with one or two such constants respectively, are capable of more satisfactory verification. It is, however, believed to be the most economical hypothesis to explain the results, whose simplicity in many respects seems to justify the search for a reasonably simple inhibition mechanism.

Fluorescence studies

On addition of the enzyme to solutions of rhodamine B, the polarization of fluorescence becomes equal to the limiting value for the dye in very viscous media. This demonstrates that physical interaction occurs, such that the dye loses its freedom to rotate independently as small molecules, and becomes bound to large molecules in the esterase preparation in the rigorous sense that it shares their rotational Brownian movement. Physical combination is also indicated by a change in absorption spectrum.

The quantitative effect of dilution on the polarization verifies that the results refer to a reversible equilibrium; and the observed displacement of rhodamine from combination by ethyl acetate provides evidence that the binding is specifically related to the inhibition of the esterase activity.

The small change of absorption spectrum which results from the combination of rhodamine B with esterase is in contrast with the large change on absorption to serum albumin (Laurence, 1952), and suggests a different mechanism of binding.

The fluorescence data are analysed quantitatively in Figs. 8 and 9. For a simple combination between dye and enzyme eqn. 5 shows that these graphs should be linear, with slope $1/c$, and intercept K/c when $C_F = 0$. (K is the dissociation constant of the combination, and c the molar concentration of binding centres in the stock enzyme solution.) Results of an identical form would be expected from the proposed inhibition mechanism. Although the dye combines at two points on the enzyme, with different dissociation constants, the two compounds EI and EI′ must always be present in a constant concentration ratio, $[\mathrm{EI}]/[\mathrm{EI}'] = K_1/K_i$, and the observed values of p_B and R will refer to this equilibrium mixture of EI and EI′. Eqn. 5 will therefore apply, with $K = 1/(1/K_1 + 1/K_i)$. The further combination $\mathrm{EI}' + \mathrm{I} \rightleftharpoons \mathrm{EI}_2$, will not be observed under the conditions of the polarization experiments, because dye concentrations are here very low. Since

$$[\mathrm{E}]\,[\mathrm{I}]^2/[\mathrm{EI}_2] = K_1K_2, \quad \text{or} \quad [\mathrm{EI}_2]/[\mathrm{E}] = C_F^2/K_1K_2,$$

and K_1K_2 was found to be $5{\cdot}3 \times 10^{-8}\ \mathrm{M}^2$ while C_F was not greater than 3×10^{-5} M, $[\mathrm{EI}_2]/[\mathrm{E}] < 0{\cdot}017$. That is, the concentration of EI_2 must always have been less than 1·7 % of that of the free enzyme, and an even smaller proportion of the total enzyme present, so that eqn. 5 cannot have been appreciably disturbed by formation of EI_2.

Figs. 8 and 9 show that, in experiments with different enzyme concentrations, $[e_a]\,C_F/C_B$ was approximately independent of $[e_a]$ and was a function of C_F only, as in eqn. 5. The results are also consistent with a linear relation between $[e_a]\,C_F/C_B$ and C_F. But the straight line drawn gives constants very different in magnitude from those expected. Its slope is $3{\cdot}6 \times 10^4\,\text{M}^{-1}$, giving $c = 0{\cdot}28 \times 10^{-4}\,\text{M}$; and its intercept at $C_F = 0$ is 0·027, so that

$$K = 0{\cdot}075 \times 10^{-5}\,\text{M},$$

which is only 0·031 × the value of

$$K = 1/(1/K_1 + 1/K_i) = 2{\cdot}4 \times 10^{-5}\,\text{M}$$

found from the inhibition data.

In the inhibition by di*iso*propyl phosphorofluoridate (Boursnell & Webb, 1949), 1 g. atom of phosphorus was bound/10^5 g. protein, corresponding to a value of $2{\cdot}2 \times 10^{-4}\,\text{M}$ for the concentration of phosphorus-binding centres in the stock enzyme solution. This is 8 times the concentration of rhodamine-binding centres found above, while it might be expected that either one or two molecules of dye would be bound for every phosphorus atom. These discrepancies are difficult to interpret. It would be possible to explain the fluorescence results without conflict with the kinetic data if, in addition to the enzymic component of K value $2{\cdot}4 \times 10^{-5}\,\text{M}$, there was also present in the enzyme preparation a small concentration of an impurity which adsorbed the dye very strongly. Then at low values of C_F, the impurity would be responsible for almost all the observed binding, while at higher C_F values the impurity would become saturated with the dye and a progressively larger fraction of the bound rhodamine would be in combination with the enzymic component.

This hypothesis is made improbable, however, by the experiment which demonstrated the displacement of the dye from combination by ethyl acetate. In this experiment C_F was initially only $2{\cdot}5 \times 10^{-7}\,\text{M}$; nearly all the bound dye should therefore have been in combination with the impurity, and the ester should not have had any marked effect. In fact it displaced the dye almost completely, suggesting that the dye was combined at the esterase centres even at the lowest C_F values used.

It has been assumed throughout the analysis of the fluorescence results that the dynamic interconversion between bound and free dye was not rapid enough to interfere with their fluorescence. Weber (1948), in an analysis of the quenching of fluorescence by collision and by complex formation, shows that, unless the mean life of a fluorescent species is much longer than the lifetime of its excited state, the polarization of its fluorescence is not constant, but depends on its mean life and hence on its concentration and that of other species present. The assumption has thus been made that the mean life of both bound and free dye was much greater than 2×10^{-9} sec., the lifetime of the excited state of rhodamine B (Weber, 1948). The following argument shows that the assumption was justified, so that no explanation of the fluorescence results is provided by this consideration.

In an equilibrium of the type, $\text{E} + \text{I} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{EI}$, the mean life of the species EI is $1/k_2$, and that of the species I is $1/k_1[\text{E}]$, which at equilibrium is equal to $[\text{I}]/[\text{EI}]\,k_2$. Since C_F/C_B was never much less than 1, the assumption may be expressed as

$$1/k_2 \gg 2 \times 10^{-9}\ \text{sec.},$$

or since the dissociation constant K is equal to k_2/k_1, $k_1 \ll 0{\cdot}5 \times 10^9/K\ \text{sec.}^{-1}$. Now k_1 is the velocity constant of a bimolecular reaction, which cannot be larger than a reasonable collision frequency; so that if K is much less than about 0·01 M, the assumption is certainly valid. Since appreciable inhibition was observed with $2{\cdot}5 \times 10^{-5}\,\text{M}$ rhodamine, at least one K value for this system must be much lower than 0·01 M, whatever the detailed mechanism of the inhibition. If several types of combination occurred simultaneously, the assumption might break down for one with a very high K value; but the proportion of bound dye present in this form would necessarily be extremely small, so that no disturbance of the observed polarization would result.

It remains possible that the interactions between rhodamine and esterase which occurred in the fluorescence studies were quantitatively different from those taking place in the inhibition work, due to the differences between the experimental conditions in the two types of experiment. While temperature and potassium chloride concentrations were identical, and phosphate concentrations differed only slightly, the enzyme concentrations of the fluorescence work were about 1000 × greater than those of the kinetic studies, and dye concentrations about 100 × smaller. For instance, a complex of the form E_2I could have been formed under the conditions of the fluorescence work, although it was not apparent in the inhibition studies.

SUMMARY

1. The inhibition of horse-liver esterase by the fluorescent dye rhodamine B has been studied kinetically; and the interaction between enzyme and dye has been investigated by measurements of the intensity and polarization of fluorescence.

2. The inhibition is rapidly established and reversible, and competitive with respect to substrate. At constant substrate and dye concentrations, the percentage inhibition is independent of enzyme and phosphate concentrations.

3. The relation between the dye concentration and the apparent value of the Michaelis constant is not linear. It resembles the function entailed by the 'apparent competitive' inhibition mechanism of Segal *et al.* (1952), but on analysis is found to deviate significantly from it.

4. An inhibition mechanism is proposed which accounts for the experimental results, including the relation between the inhibitions of the hydrolyses of two different substrates.

5. Distribution experiments and freezing-point measurements show that rhodamine B is monomeric in the aqueous solutions of pH 7·4 used for the enzyme studies.

6. Measurements of the polarization of fluorescence demonstrate the reversible combination of rhodamine with large molecules in the enzyme preparation. The combination is accompanied by a change in absorption spectrum and an increase in fluorescent intensity.

7. Displacement of the dye from combination by ethyl acetate gives evidence that the binding is specifically related to the esterase inhibition.

8. The fluorescence data are analysed quantitatively, and their relation to the inhibition results is discussed.

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The Oxidation of Indolyl-3-Acetic Acid by Waxpod Bean Root Sap and Peroxidase Systems

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The presence in extracts of certain plant tissues of systems which oxidize indolyl-3-acetic acid (IAA) is well established (e.g. by Tang & Bonner, 1947, 1948; Wagenknecht & Burris, 1950; Gortner & Kent, 1953). The enzyme system present in extracts of etiolated pea epicotyls has been most studied, and it has been suggested that the system consists of a flavoprotein which gives rise to hydrogen peroxide and a peroxidase which catalyses the oxidation of IAA by hydrogen peroxide (cf. Galston, Bonner & Baker, 1953). The enzyme in extracts of waxpod bean roots has received little attention, although according to Wagenknecht & Burris (1950) such extracts show greater activity towards IAA than extracts of etiolated pea epicotyls. Wagenknecht & Burris (1950) have shown that the rate of oxidation of IAA by dialysed extracts of waxpod bean roots is increased in the presence of suitable amounts of Mn^{2+}. It has been shown by Galston *et al.* (1953) that horseradish peroxidase preparations catalyse the oxidation of IAA in the absence of added hydrogen peroxide, and Kenten (1953) has reported that the rate of this oxidation is stimulated in the presence of Mn^{2+}. These facts suggested that the system peroxidase–Mn^{2+} might be responsible for the activity of extracts of waxpod bean roots towards IAA, and the present work was undertaken to investigate this possibility.

MATERIALS AND METHODS

Plant sap. The waxpod beans (*Phaseolus vulgaris*, L., var. Sutton's Golden Waxpod) were glasshouse-grown in a John Innes potting compost. They were harvested 4–8 weeks after sowing. The rest of the plants were found growing wild or in gardens in the neighbourhood of Harpenden. The plant material was washed free from soil, and either ground in a mortar with sand or passed through a domestic meat mincer. The pulp was squeezed by hand through strong cotton cloth, the sap centrifuged for 15–20 min. at 6000 rev./min., the precipitate discarded and the supernatant stored at 0°.

Peroxidase. Peroxidase preparations were made from horseradish root by the method of Kenten & Mann (1954). The purpurogallin number (P.N.) of the preparations was estimated by the method of Keilin & Hartree (1951). The most highly purified preparation used had a P.N. of 1040 and a haemin content of 1·12%. In the text the peroxidase activity of some preparations is given as enzyme units (E.U.) (Keilin & Hartree, 1951). An amount of preparation containing 1 E.U. would form 1 g. of purpurogallin under Keilin & Hartree's conditions. With a dry preparation the number of E.U. = P.N. × wt. of the preparation in g.

Catalase. This was prepared from horse liver by a method based on that of Agner (1938) and the activity of the preparation was estimated as described previously (Kenten & Mann, 1952). The Katalasefahigkeit (Kat.f.) was 5000. Using the equation of Chance & Herbert (1950) that Kat.f. $= 520k_1'/M$, where M is the molecular weight of catalase (230000), the velocity constant of the preparation, $k_1' = 2{\cdot}2 \times 10^6 \text{M}^{-1}$ sec.$^{-1}$. For each experiment a sample of the preparation was suspended in 0·5% (w/v) NaCl (10 mg./ml.) and the insoluble material removed by centrifuging.

D-*Amino acid oxidase.* This was prepared from sheep kidney. The method of Negelein & Brömel (1939) was followed as far as the first precipitation with $(NH_4)_2SO_4$. This precipitate was dissolved in 0·003 M pyrophosphate, pH 8·5, and reprecipitated by the addition of 30 g. $(NH_4)_2SO_4$/100 ml. The precipitate was separated by centrifuging and dissolved in 0·003 M pyrophosphate at pH 8·3. 1 ml. of this preparation catalysed the uptake of approximately 100 μl. O_2/10 min. with DL-alanine as substrate under the conditions of Negelein & Brömel (1939).

IAA solutions. Indolyl-3-acetic acid solutions were adjusted to the appropriate pH (usually pH 6) with 5% (w/v) NH_3 and stored in the dark at 0°.

Manometric experiments. These were carried out in the Warburg apparatus at 25°. The volume of the reaction mixture was 3 ml. and KOH was present in the centre cup unless otherwise stated. The formation of CO_2 was measured by Warburg's direct method (Dixon, 1943). For all the experiments the apparatus was covered by a hood; this considerably reduced the amount of light reaching the reaction mixtures. While light was not completely excluded, the intensity of illumination at the surface of the bath was always less than 1 ft. candle.

EXPERIMENTAL AND RESULTS

Manometric studies of the oxidation of IAA by waxpod bean root sap

Wagenknecht & Burris (1950) showed that waxpod bean root sap lost about one-third of its activity towards IAA after dialysing for 24 hr. Using suspensions of ground pea epicotyls, Galston & Baker (1951) reported large increases in activity following dialysis, whereas Goldacre, Galston & Weintraub (1953) found a partial loss of activity, which was restored by the addition of dialysate. In the present work with waxpod bean root sap a loss of activity towards IAA has always been found following dialysis, and the results suggest that this decrease is due to the loss of a thermostable fraction. By ultrafiltration the IAA-oxidizing system in waxpod bean root sap can be separated into a thermolabile fraction and a thermostable fraction. The thermolabile fraction alone oxidizes IAA but the rate of oxidation is increased in the presence of the thermostable fraction. The thermolabile fraction can be replaced by horseradish peroxidase preparations.

The effect of ultrafiltration. Centrifuged waxpod bean root sap (15 ml.) was filtered for 24 hr. in an ultrafiltration apparatus of the type described by Paterson, Pirie & Stableforth (1947). In this time nearly all the liquid passed through, leaving a small pasty residue inside the cellophan sac. The residue was suspended in 15 ml. of water. The O_2 uptake brought about by the addition of these fractions to IAA is shown in Fig. 1. While the ultrafiltrate alone showed negligible activity and the residue alone about half the activity of the sap, together they gave an activity only slightly less than the sap. The activating effect of the ultrafiltrate was not affected by previous heating at 100° for 15 min., whereas only slight activity remained in the residue after similar heating.

Replacement of waxpod bean root residue with horseradish peroxidase preparations. The waxpod bean root residue has high peroxidase activity;

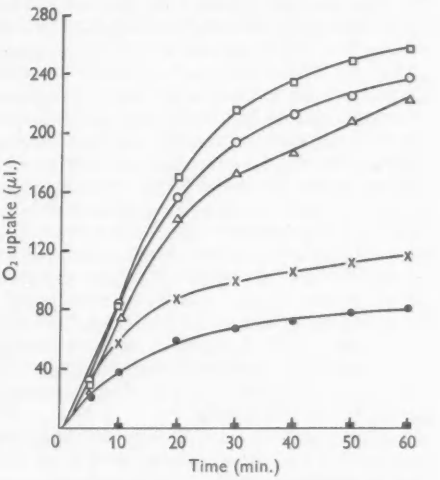

Fig. 1. Effect of ultrafiltration on the rate of oxidation of IAA by waxpod bean root sap. Reaction mixtures in 0·067 M phosphate at pH 6 and 0·3 ml. 0·05 M IAA added from the side arm at zero time, temp. 25°; gas phase, air. ○—○, 0·5 ml. sap.; × — ×, 0·5 ml. residue (0·17 E.U./ml.); ■—■, 0·5 ml. ultrafiltrate; △—△, 0·5 ml. residue + 0·5 ml. ultrafiltrate; ●—●, 85 μg. horseradish peroxidase of P.N. 1040; □—□, 85 μg. horseradish peroxidase of P.N. 1040 + 0·5 ultrafiltrate. The O_2 uptakes are corrected for the uptake in absence of IAA.

under the standard conditions (Keilin & Hartree, 1951) with pyrogallol as substrate, the activity of different preparations varied from 0·045 to 0·19 E.U./ml. The effect of replacing residue with an amount of a horseradish peroxidase preparation was accordingly tested. The O_2 uptakes are shown in Fig. 1. In this experiment the amount of horseradish peroxidase used gave a lower rate of uptake than did waxpod bean root residue, but its activity was raised above that of sap by adding ultrafiltrate.

This experiment suggests that peroxidase together with some thermostable factor or factors of small molecular weight form a powerful IAA oxidase system. However, since Galston *et al.* (1953) have suggested that the IAA-oxidizing system in extracts of pea epicotyls consists of a flavoprotein–peroxidase system, it might be argued that the peroxidase preparation used was contaminated with flavoprotein or with the protein moiety of a flavoprotein, and that the function of the ultrafiltrate was to provide flavoprotein substrate or flavins respectively, and that in effect a flavoprotein–peroxidase system was being reconstituted. The evidence obtained by Kenten & Mann (1954) suggests that the peroxidase preparation of P.N. 1040 which was used is about 90% peroxidase. Furthermore, whereas riboflavin or flavin mononucleotide could be readily detected at a concentration of 0·1 μg./ml. by the appearance of a yellow fluorescence in ultraviolet light, when a solution of horseradish peroxidase of P.N. 1040 (1 mg./ml.) was heat denatured and centrifuged, no yellow fluorescence appeared when the supernatant was exposed to ultraviolet light. Galston *et al.* (1953) made use of the technique suggested by Warburg & Christian (1938) for the removal of the prosthetic group of flavoproteins. By treating an IAA oxidase preparation from pea epicotyls with $(NH_4)_2SO_4$ at pH 2·5 they obtained two fractions; a protein which precipitated at 0·22 saturation, and a soluble fraction containing peroxidase and flavins. Neither of these fractions was significantly active towards IAA, but when mixed they readily oxidized IAA. The peroxidase which was used to replace the non-filtrable residue in the experiment described above was accordingly treated in a similar fashion.

A solution of peroxidase, 10 ml., containing 1 mg. peroxidase of P.N. 1040/ml., was cooled in ice water to 0°, and saturated $(NH_4)_2SO_4$ in water at 0° and 0·1 N-H_2SO_4 added until the pH was 2·5, and the solution 0·25 saturated with respect to $(NH_4)_2SO_4$ at 0°. After 30 min. at 0° the solution was centrifuged but yielded only a trace of grey precipitate. The supernatant was then adjusted to pH 6 with N-NaOH after the addition of 1 ml. 0·2 M-phosphate, pH 6, and dialysed overnight against water. The dialysed solution was centrifuged and a trace of yellow precipitate was obtained and discarded. Activity measurements on the supernatant showed that 75% of the peroxidase activity had been recovered. The activity of this acid–$(NH_4)_2SO_4$–treated peroxidase was compared with the activity of the untreated peroxidase and the results are given in Table 1. The results obtained with a crude horseradish peroxidase of P.N. 130 are also included in this table. There is little difference between the activities of the three peroxidases in the presence of the ultrafiltrate but in the absence of the ultrafiltrate the acid-treated peroxidase was slightly less active than was the untreated peroxidases.

Under the conditions of Table 1 the presence of 5 or 50 μg. of either riboflavin or flavin mononucleotide did not affect the rate at which the acid–$(NH_4)_2SO_4$-treated peroxidase oxidized IAA. A preparation of flavin-adenine dinucleotide (FAD) was not available, but the addition of 1 ml. boiled D-amino acid oxidase preparation (the prosthetic group of this oxidase is FAD) was also without effect on the rate of oxidation of IAA by the treated peroxidase.

The results suggest that the activity of ultrafiltrate is not due to the presence of flavin compounds. Also, the fact that the $(NH_4)_2SO_4$–acid treatment has comparatively little effect on the activity of the purified peroxidase suggests that the observed activity is due to peroxidase and not to peroxidase plus flavoprotein.

Effect of varying the concentration of bean root residue, horseradish peroxidase and ultrafiltrate. The effect of varying the amounts of bean root residue, peroxidase and ultrafiltrate on the rate of O_2 uptake by solutions of IAA is shown in Table 2 and Fig. 2.

In the experiment given in Table 2 the activity of the bean root residue alone is less than that of an amount of highly purified peroxidase having the same peroxidase activity. This was not invariably the case; the activity of different batches of bean

Table 1. *Effect of acid–ammonium sulphate treatment on the activity of a highly purified horseradish peroxidase preparation*

Reaction mixtures consisted of: in the main vessel horseradish peroxidase preparation equivalent to 0·08 E.U., 0·067 M phosphate at pH 6, and, where present, 0·5 ml. waxpod bean root ultrafiltrate. After equilibration, 0·3 ml. of 0·05 M IAA was added from the side arm. The O_2 uptakes obtained when IAA or peroxidase was omitted were negligible.

Peroxidase	P.N.	Uptake of O_2 (μl. in 15 min.) Alone	Ultrafiltrate added
Horseradish	1040	62·5	155·5
Horseradish, acid–$(NH_4)_2SO_4$ treated	1040	45·5	139·5
Horseradish	130	57·5	140·5

Table 2. *Effect of varying the amount of peroxidase and waxpod bean root residue in the presence of a constant amount of ultrafiltrate*

Reaction mixtures consisted of varying amounts of horseradish peroxidase of P.N. 1040 and bean root residue, and where present, either 0·4 or 0·8 ml. ultrafiltrate in 0·067 M phosphate at pH 6. After equilibration, 0·3 ml. 0·05 M IAA was added from the side arm. All the O_2 uptakes recorded were obtained using the same batch of ultrafiltrate. The O_2 uptakes are corrected for the blank O_2 uptakes in mixtures from which IAA was omitted. These were less than 7 μl. in 30 min.

Horseradish peroxidase (E.U. $\times 10^{-3}$ present)	Waxpod bean root residue (E.U. $\times 10^{-3}$ present)	O_2 uptake (μl./30 min.) No ultrafiltrate	Ultrafiltrate added (ml.) 0·4	Ultrafiltrate added (ml.) 0·8	Increase due to presence of ultrafiltrate (ml.) 0·4	Increase due to presence of ultrafiltrate (ml.) 0·8
0·5	—	0	23·5	45·5	23·5	45·5
2·0	—	0	61	88	61	88
8·0	—	3	79·5	132	76·5	129
16	—	9·5	113·5	174	104	164·5
32	—	29·5	128·5	199	99	169·5
64	—	57·5	160	228·5	102·5	171
—	2·0	1·5	4·5	12·5	3	11
—	8·0	0	18	33·5	18	33·5
—	16	0	37·5	58·5	37·5	58·5
—	32	7	61	92	54	85
—	64	20	85	138	65	118

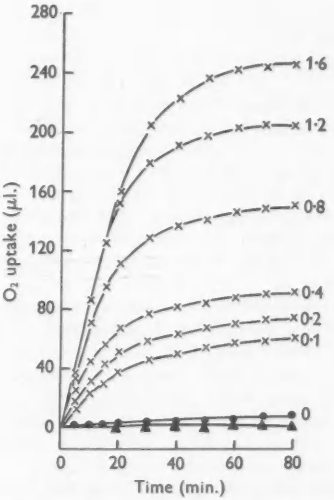

Fig. 2. Effect of variation in the concentration of ultrafiltrate on the rate of oxidation of IAA by a horseradish peroxidase preparation of P.N. 1040. Reaction mixtures consisted of 8 μg. horseradish peroxidase of P.N. 1040, 0·067 M phosphate at pH 6 and varying amounts of waxpod bean root ultrafiltrate (ml. added denoted by the number to the right of each curve). After equilibration 0·3 ml. 0·05 M IAA was added from the side arm. ●—●, peroxidase; ▲—▲, 1·6 ml. ultrafiltrate; ×—×, peroxidase + ultrafiltrate.

root residue varied considerably, and sometimes had a greater activity than an equivalent amount of horseradish peroxidase. One of the reasons for this variation may have been variation in the degree of contamination of the bean root residue by the natural activating factor(s).

The ultrafiltrate alone was always inactive (< 10 μl. O_2/ml./hr. with IAA), and while it invariably increased the activity of bean root residue and horseradish peroxidase towards IAA, its capacity in this respect varied widely. With small amounts of bean root residue alone or peroxidase alone the activity is negligible, but if suitable amounts of ultrafiltrate are also present the oxidation of IAA proceeds readily (Fig. 2). With any one preparation of ultrafiltrate, and using an amount of horseradish peroxidase equivalent to the peroxidase activity of bean root residue, the system, residue + ultrafiltrate, was less active than horseradish peroxidase + ultrafiltrate. This suggests that waxpod bean root peroxidase catalyses the oxidation of IAA less readily than does horseradish peroxidase.

In the presence of a constant amount of ultrafiltrate (0·4 or 0·8 ml.), increasing the amount of horseradish peroxidase from 0–16 μg. increases the activity of the system, but with amounts of peroxidase greater than 16 μg. the further increase in activity can be accounted for by the activity of peroxidase alone (Table 2). Increasing the amount of ultrafiltrate in the presence of a constant amount of horseradish peroxidase increases the activity of the system (Fig. 2), although under the conditions of the experiment there is little difference in the initial rate of oxidation in those cases where 1·2 and 1·6 ml. of ultrafiltrate are present. It can be seen that the effect of the ultrafiltrate is largely restricted to the initial 30–40 min. of the reaction period, after which the reaction practically ceases, although with the smaller amounts of ultrafiltrate less than half the added IAA has been oxidized. The effect of

making a second addition of ultrafiltrate or peroxidase after the initial rapid phase of the reaction was complete is shown in Table 3. It is clear that while a second addition of ultrafiltrate has only a comparatively small effect, a second addition of peroxidase causes a further O_2 uptake only slightly less than that obtained initially. This suggests that the peroxidase, not the ultrafiltrate factors, is inactivated during the oxidation of IAA. Incubation of peroxidase with IAA for 1 hr., under conditions where little oxidation of IAA takes place, does lead to small loss of peroxidase activity, since on adding ultrafiltrate the reaction proceeds at a slightly slower rate than that found with mixtures in which the peroxidase has not been incubated with IAA. Peroxidase estimations on initial and final reaction mixtures of similar composition to those used in the experiments given in Table 3, showed that where peroxidase had been incubated with IAA there was only a small loss of peroxidase activity (about 30 %) in the solution, but where bean root ultrafiltrate was also present and a large amount of IAA had been oxidized there was a large loss of peroxidase activity (about 90–95 %). Control reaction mixtures showed that 8 μg. peroxidase incubated at 25° with phosphate at pH 6 either in the presence or the absence of the ultrafiltrate lost about 15 % of its activity in 1 hr. It would appear, therefore, that the activity of the ultrafiltrate is not a protection of the peroxidase from inactivation during the oxidation of IAA. The loss of peroxidase activity would appear to be a consequence of the oxidation of IAA and could be due to the formation of toxic or inhibiting oxidation products.

Although the results with bean root residue and horseradish peroxidase differ quantitatively, they are essentially similar, and suggest that the activity of the waxpod bean root sap towards IAA could be due to peroxidase plus thermostable factors.

Table 3. *Loss of peroxidase activity during the oxidation of IAA*

Reaction mixtures consisted of: in the main vessel 0·3 ml. 0·05M IAA in 0·067M phosphate at pH 6 and where present 0·3 ml. waxpod bean root ultrafiltrate. After equilibration 8 μg. horseradish peroxidase of P.N. 1040 in 0·1 ml. were added from the side arm. After 1 hr. a further addition of peroxidase or ultrafiltrate was made from the second side arm as shown below. Blank O_2 uptakes with IAA omitted or IAA + ultrafiltrate were less than 5 μl. in 2 hr.

Components present with IAA	Uptake of O_2 in first hr. (μl.)	After 1 hr. add	Uptake of O_2 in second hr. (μl.)
Peroxidase	3	—	3·5
	2·5	Peroxidase	4·5
	4·5	Ultrafiltrate	94
Peroxidase + ultrafiltrate	109	—	7
	104·5	Ultrafiltrate	18
	111	Peroxidase	74

The nature of the thermostable factors. Studies of the effect of Mn^{2+} on the peroxidase-catalysed oxidation of IAA by O_2, which will be described later in this paper, show that while Mn^{2+}, under suitable conditions, increases the rate of oxidation, this effect is small compared with that of waxpod bean root ultrafiltrate. The activity of ultrafiltrate cannot, therefore, be due to the presence of Mn^{2+} alone. Goldacre *et al.* (1953) have shown that certain phenols, when present in small amounts, bring about large increases in the activity of pea epicotyl brei towards IAA. These authors, while not excluding the possibility that the active phenols were operating in a catalytic manner by a reversible oxidation–reduction, suggested that they might serve as cofactors. They found that a large number of monophenols, particularly chloromonophenols, were active, but with dihydric phenols no activity could be demonstrated. In this connexion it is of interest that Kenten & Mann (1950) found that monohydric phenols but not di- or tri-hydric phenols (with the exception of resorcinol) were active as carriers in the peroxidase-catalysed oxidation of Mn^{2+} by H_2O_2. The results of Kenten & Mann (1950) suggested that it was the intermediate and not the final oxidation products of the phenols which were active, since prior incubation with peroxidase and H_2O_2 destroyed the activity of the phenols. If the active factor in the ultrafiltrate is acting catalytically, being oxidized by peroxidase and reduced by IAA, then in the absence of IAA it might be oxidized to an irreversible oxidation product.

This was tested in reaction mixtures consisting of: ultrafiltrate, 2·5 ml.; 100 μg. horseradish peroxidase of P.N. 1040; 0·05M-H_2O_2, 0·4 ml.; and water to a final volume of 4 ml. Control mixtures were made up from which either peroxidase, or H_2O_2, or both peroxidase and H_2O_2 were omitted. After 3 hr. at room temperature 20 μg. catalase in 0·1 ml. were added to decompose the residual H_2O_2 and 10 min. later the solutions were heated at 100° for 20 min. to inactivate the peroxidase and catalase. After cooling, portions of the solutions were tested manometrically under the conditions given in Table 4.

The results show that while the treatment of the ultrafiltrate with H_2O_2 alone or peroxidase alone has little effect on its activity, the treatment with both peroxidase and H_2O_2 present has reduced the O_2 uptake obtained in the first 30 min. by about 60 %. That this effect was not due to the formation of inhibiting substances is shown by the O_2 uptake obtained when solutions from treatments 1 and 2 were both present (Table 4). Increasing the time of the treatment with peroxidase and H_2O_2 or in-

Table 4. *Loss of activity of ultrafiltrate, 2:4:6-trichlorophenol and p-chlorophenol by treatment with peroxidase plus H_2O_2*

Treatments with peroxidase and H_2O_2 as described in the text. Manometric reaction mixtures consisted of: in the main vessel 1 ml. of the treated ultrafiltrate solutions or 0·8 ml. treated chlorophenol solutions or 0·6 or 0·06 ml. untreated ultrafiltrate, 16 μg. horseradish peroxidase of P.N. 1040 and phosphate at pH 6, 0·067 M (ultrafiltrate experiment) and 0·035 M (chlorophenol experiment). After equilibration, 0·3 ml. 0·05 M IAA was added from the side arm. The O_2 uptakes are corrected for the uptake due to peroxidase in the absence of the chlorophenols or ultrafiltrate which was 16 μl.

Treatment or addition	Uptake of O_2 (μl. in 30 min.)		
	Ultrafiltrate	2:4:6-Trichlorophenol	*p*-Chlorophenol
None	123·5	65	98
Peroxidase + H_2O_2	49	6·5	18
H_2O_2 alone	134·5	61·5	76·5
Peroxidase alone	123	72·5	97·5
Equal volumes of solutions from treatments 1 and 2 mixed and 2 ml. (ultrafiltrate experiment) or 1·6 ml. (chlorophenol experiment) tested	161	77	109
Ultrafiltrate 0·6 ml.	124·5	—	—
Ultrafiltrate 0·06 ml.	35	—	—

Table 5. *Activity of phenols, aromatic amines and maleic hydrazide*

Reaction mixtures consisted of: in the main vessel 16 μg. horseradish peroxidase of P.N. 1040 and the substances tested in 0·067 M phosphate at pH 6. After equilibration 0·3 ml. 0·05 M IAA was added from the side arm. The O_2 uptakes are corrected for the uptake due to the action of peroxidase alone on IAA. These blank uptakes varied between 10 and 16 μl. O_2. Blank uptakes when IAA was omitted were negligible.

Concentration (M) ...	Increased uptake in 30 min. (μl. O_2)			
Substance	5×10^{-3}	10^{-3}	10^{-4}	10^{-5}
Phenol	38	82·5	42·5	14
p-Chlorophenol	14	62·5	84	44
4-Methylumbelliferone	—	109·5	58	26
Resorcinol	4·5	32·5	79·5	20·5
Aniline	25	45·5	37	15·5
Maleic hydrazide	52·5	8	0	0

Catechol, hydroquinone, pyrogallol and *p*-phenylenediamine inhibited when present in concentrations of 5×10^{-3}–10^{-5} M.

creasing the amounts of peroxidase and H_2O_2 did not result in a significantly larger decrease in the activity of ultrafiltrate. With other batches of ultrafiltrate the loss of activity following treatment with peroxidase plus H_2O_2 varied between 65 and 50 %, based on the O_2 uptake. However, the increase in O_2 uptake is not directly proportional to the amount of ultrafiltrate present. The amount of ultrafiltrate present in the portions of treated ultrafiltrate solutions tested manometrically is equivalent to 0·6 ml., and it is shown in Table 4 that reducing the amount of ultrafiltrate from 0·6 to 0·06 ml. reduces the O_2 uptake by only 70 %. Thus, the results suggest that a large part of the activity of the ultrafiltrate is due to the presence of peroxidase substrates which give rise to inactive products when oxidized by peroxidase and H_2O_2. It is of course possible that the activity remaining in the ultrafiltrate after treatment with peroxidase and H_2O_2 is also due to peroxidase substrates which are not irreversibly oxidized under the conditions used.

The effect of peroxidase substrates on the oxidation of IAA by horseradish peroxidase preparations. In agreement with the suggestion that peroxidase substrates are partially responsible for the activity of bean root ultrafiltrate, it was found that certain monophenols, and also aniline, increased the rate of oxidation of IAA by peroxidase. In addition to a number of known peroxidase substrates, maleic hydrazide, 4-methylumbelliferone and *p*-chlorophenol were also tested, since these compounds are known to increase the activity of pea epicotyl preparations towards IAA (Goldacre *et al.* 1953; Andreae & Andreae, 1953). At the concentration used, resorcinol, aniline, maleic hydrazide and the monohydric phenols increased the activity of the system, while the dihydric and trihydric phenols and *p*-phenylenediamine decreased the activity (Table 5). The results suggest that with *p*-chlorophenol, resorcinol and aniline there is an optimum concentration, and that if these compounds are present in too high a concentration little or no increase in activity is observed. With the exception of maleic hydrazide, 4-methylumbelliferone and *p*-chlorophenol, all of the compounds tested in the experiments recorded in Table 5 are already known to be peroxidase substrates. Colorimetric evidence was obtained that both 4-methylumbelliferone and *p*-chlorophenol are peroxidase substrates, since when peroxidase was added to solutions of these compounds in dilute H_2O_2 at pH 6, the umbelliferone solution went rapidly turbid and finally a white insoluble oxidation product separated, while with *p*-chlorophenol coloured oxidation products

were formed. Also, as will be shown later, the activating capacity of *p*-chlorophenol on the peroxidase-catalysed oxidation of IAA by O_2 is largely destroyed by prior incubation with peroxidase plus H_2O_2. With maleic hydrazide, however, no visual evidence of oxidation by peroxidase plus H_2O_2 at pH 6 was obtained, and its activating capacity was not affected by this treatment.

It is clear from the results that certain peroxidase substrates, when present in catalytic amounts, are capable of increasing the rate of oxidation of IAA by peroxidase. Furthermore, those peroxidase substrates which are active in this respect, namely, the monohydric phenols, aniline, and resorcinol, are also active as carriers in the peroxidase-catalysed oxidation of Mn^{2+} by H_2O_2 (Kenten & Mann, 1950; Kenten & Mann, unpublished observations), while catechol, hydroquinone, pyrogallol and *p*-phenylenediamine, which inhibit the oxidation of IAA, are inactive in the oxidation of Mn^{2+}.

Loss of activity of 2:4:6-*trichlorophenol and* p-*chlorophenol following treatment with peroxidase plus* H_2O_2. Goldacre *et al.* (1953) found that 2:4:6-trichlorophenol activated the oxidation of IAA by dialysed pea epicotyl brei. They considered that this phenol was unlikely to be functioning in an oxidation–reduction system, since it appeared to be incapable of reversible oxidation via an *o*- or *p*-quinone. It was, therefore, considered of interest to test whether prior incubation with H_2O_2 and peroxidase would destroy the activating capacity of this phenol. In addition *p*-chlorophenol was also tested.

The experimental procedure was similar to that previously described for the ultrafiltrate, except that the reaction was allowed to proceed overnight (approximately 18 hr.). The complete reaction mixture consisted of: 0·002 M 2:4:6-trichlorophenol or *p*-chlorophenol, 1 ml.; 100 μg. horseradish peroxidase of P.N. 1040 and 0·05 M-H_2O_2, 0·5 ml., in a total volume of 5 ml., 0·04 M with respect to phosphate at pH 6.

The results (Table 4) show that the treatment, with both peroxidase and H_2O_2 present, has largely destroyed the activity of the phenols. Using *p*-chlorophenol and varying the amount of peroxidase from 50 to 200 μg. and the H_2O_2 from 1 to 0·2 ml., 0·05 M, did not result in a significantly larger decrease in activity than that given in Table 4. The small residual activating capacity of the phenol solutions after treatment with peroxidase plus H_2O_2 is of interest in connexion with the failure to completely inactivate ultrafiltrate by such treatment.

The inhibiting effect of certain peroxidase substrates and the reversal of such inhibition by the addition of H_2O_2. Small amounts of catechol, hydroquinone, pyrogallol, *p*-phenylenediamine and ascorbic acid strongly inhibit the oxidation of IAA by peroxidase plus ultrafiltrate or *p*-chlorophenol. It is shown in Table 6 that the addition of a small amount of H_2O_2 reverses this inhibition. In order to cause complete inhibition in the experiment with the ultrafiltrate it was necessary to use a higher concentration of pyrogallol ($3·3 \times 10^{-5}$ M) than in the experiment with *p*-chlorophenol, where 10^{-5} M was sufficient. Even so, with the ultrafiltrate, pyrogallol only inhibited initially. At the end of 1 hr. an O_2 uptake started and then proceeded with increasing velocity. With *p*-chlorophenol and the lower concentration of pyrogallol no O_2 uptake was observed after 1·5 hr. It is possible that the ultrafiltrate contains a factor which accelerates the autoxidation of pyrogallol or that the ultrafiltrate-containing system produces peroxide more readily than does the *p*-chlorophenol system. Using 10^{-4} M ascorbic acid as an inhibiting peroxidase substrate in place of pyrogallol, and working in citrate at pH 6 to reduce loss of ascorbic acid by autoxidation, essentially similar results were obtained, except that with the ultrafiltrate-containing system at this concentration of ascorbic acid, in the absence of added H_2O_2, the reaction had not started after a 2 hr. period.

Experiments with horseradish root sap. Horseradish root sap, although rich in peroxidase, does not catalyse the oxidation of IAA by O_2. However, if the sap is treated with a suitable amount of H_2O_2

Table 6. *Reversal of pyrogallol inhibition by addition of* H_2O_2

A. The complete reaction mixture consisted of: in the main vessel 16 μg. horseradish peroxidase of P.N. 1040, 0·001 M *p*-chlorophenol, 0·3 ml., and 0·0001 M pyrogallol, 0·3 ml. in 0·067 M phosphate at pH 6. After equilibration, 0·05 M IAA, 0·3 ml., and 0·001 M-H_2O_2, 0·2 ml., were added from the side arm.

B. The complete reaction mixture consisted of the same quantities of reagents as in *A* except that 0·6 ml. ultrafiltrate was used in place of *p*-chlorophenol solution, the amount of pyrogallol was 1 ml. 0·0001 M- and 0·2 ml. 0·002 M-H_2O_2 was present in the side arm with the IAA. The composition of the control reaction mixtures is shown below.

Components present with IAA	Uptake in 30 min. (μl. O_2)
A. With *p*-chlorophenol	
Peroxidase	16
Peroxidase + H_2O_2	15·5
Peroxidase + *p*-chlorophenol	104
Peroxidase + *p*-chlorophenol + H_2O_2	100·5
Peroxidase + *p*-chlorophenol + pyrogallol	1·5
Peroxidase + *p*-chlorophenol + pyrogallol + H_2O_2	96·5
Peroxidase + pyrogallol + H_2O_2	7
B. With ultrafiltrate	
Peroxidase	9
Peroxidase + ultrafiltrate	143
Peroxidase + ultrafiltrate + H_2O_2	114
Peroxidase + ultrafiltrate + pyrogallol	1·5
Peroxidase + ultrafiltrate + pyrogallol + H_2O_2	123
Ultrafiltrate + H_2O_2	2·5

before testing, then it readily oxidizes IAA. This is shown in Table 7. The results suggest that peroxidase substrates are present in the sap which inhibit in a way similar to that of pyrogallol.

Sap obtained from spinach beet (*Beta vulgaris* L.) leaves and from waxpod bean leaves had peroxidase activity but did not oxidize added IAA, and attempts to demonstrate IAA oxidation after treating the saps with H_2O_2 were unsuccessful. Unlike the sap of horseradish root, which has a very low catalatic activity, these leaf saps decompose H_2O_2 very rapidly, and it may be that the added H_2O_2 was decomposed too rapidly for the oxidation of inhibiting substances to take place.

Table 7. *Oxidation of IAA by horseradish root sap treated with* H_2O_2

Mixtures containing 1·5 ml. horseradish root sap adjusted to pH 6 with N-NaOH, varying amounts (0–0·5 ml.) of 0·05 M-H_2O_2, and water to a volume of 3 ml. were stood at room temperature for 1·5 hr. Then 20 μg. catalase in 0·2 ml. were added to decompose residual H_2O_2 and 30 min. later portions of the mixtures were tested manometrically with IAA. A mixture of 1·5 ml. sap at pH 6 with 1·5 ml. water which had been shaken for 4 hr. in air at room temperature was also tested. Manometric reaction mixtures consisted of: in the main vessel 1·2 ml. portions of the treated sap in 0·033 M phosphate at pH 6. After equilibration 0·3 ml. 0·05 M IAA was added from the side arm. The O_2 uptakes are corrected for the uptakes obtained when IAA was omitted.

Treatment (ml. of H_2O_2 added)	Uptake of O_2 (μl. in 1 hr.)
0	5·5
0·1	12
0·2	113
0·4	69
0·5	48·5
Shaken 4 hr. in air	1
Equal volumes from treatments 1 and 3 mixed and 2·4 ml. tested	2

Table 8. *Effect of* Mn^{2+} *on the oxidation of IAA by different peroxidase preparations*

Reaction mixtures consisted of: peroxidase preparation equivalent to 0·05 E.U., 0·067 M phosphate at pH 6 and where present 0·1 ml. 0·001 M-$MnSO_4$. After equilibration 0·4 ml. 0·05 M IAA was added from the side arm. The O_2 uptakes when peroxidase or IAA were omitted were negligible.

		Uptake of O_2 (μl. in 90 min.)		
	P.N.	No addition	Mn^{2+} added	Increase due to Mn^{2+}
Horseradish	60	123·5	185·5	62
Horseradish	500	117	211	94
Horseradish	940	85	115	30
Horseradish	1040	89	120	31
Turnip root	70	100·5	204·5	104

Effect of catalase. The effect of catalase on the oxidation of IAA by horseradish peroxidase plus phenol was tested manometrically using 16 μg. peroxidase of P.N. 1040, 2×10^{-4} M phenol, 0·005 M IAA and 0·067 M phosphate buffer. The inhibiting effect of catalase varied with the pH, and it was necessary to use large amounts to obtain an inhibition. When 5 mg. catalase preparation were present in the reaction mixture at pH 6·5 or 7, no oxidation of IAA was observed in the experimental period of 1 hr.; but at pH 6 the presence of 5 mg. catalase caused only a short lag period (approximately 10 min.), after which the oxidation proceeded with increasing velocity and finally reached a velocity slightly greater than that of the control from which catalase was omitted. The inhibiting effect of catalase at pH 6·5 and 7, which is around the optimum for catalase action, suggests that the reaction depends on the production of H_2O_2.

Effect of manganese on the oxidation of IAA by horseradish peroxidase preparations. The effect of Mn^{2+} on the oxidation of IAA by horseradish peroxidase has been found to depend on the pH, the amount of Mn^{2+} present, the purity of the peroxidase preparation and the composition of the buffer solution.

Effect of pH. Under the conditions used (0·067 M phosphate–0·005 M IAA) the activating effect of small amounts of Mn^{2+} is greatest in the neighbourhood of pH 6, at which pH horseradish peroxidase alone catalyses the oxidation of IAA most readily.

Effect of purity of the peroxidase preparation. When a number of peroxidase preparations of varying degrees of purity were tested with Mn^{2+}, it was found that the activating effect of Mn^{2+} was least with the two most highly purified preparations used (Table 8). This suggests that the cruder preparations contain a factor which increases the effect of Mn^{2+}.

Effect of varying the concentration of Mn^{2+}. The effect of varying the concentration of Mn^{2+} on the oxidation of IAA by the peroxidase preparation of P.N. 500 in phosphate at pH 6 is shown in Fig. 3. With increasing amounts of Mn^{2+} up to about 10^{-5} M, the initial rate of O_2 uptake increases, but with further increase in Mn^{2+} there is a lag period before the oxidation begins. The length of the lag period varied in different experiments, but in general increased with increasing amounts of Mn^{2+}. Similar results were obtained when the highly purified peroxidase of P.N. 1040 was used, but here the activating effect of Mn^{2+} was less marked. It can be seen (Fig. 3) that the presence of an optimum amount of Mn^{2+} increased the rate of oxidation of IAA by 40 μg. peroxidase of P.N. 500 about 2–3 times; the effect of Mn^{2+} was no greater with small amounts of peroxidase (e.g. 8 μg. of P.N. 1040).

Since it has been shown (Fig. 2) that the rate of oxidation of IAA by 8 μg. peroxidase of P.N. 1040 is increased some 20–30 times in the presence of suitable amounts of waxpod bean root ultrafiltrate, it is clear that Mn^{2+} is much less active than is the ultrafiltrate.

Effect of the composition of the buffer solution. At pH 6 in the absence of Mn^{2+} the rate of oxidation of IAA by peroxidase was greater in pyrophosphate and phosphate–citrate media than the rate in phosphate. Under the conditions used this difference was greatest in the first 10–20 min. of the reaction where in pyrophosphate the rate of oxidation was nearly twice as fast as in phosphate, but since the rate of oxidation fell off more rapidly in pyrophosphate than in phosphate, the difference was less marked in the later stages of the reaction. However, in pyrophosphate or phosphate–citrate buffer at pH 6 the activating effect of low amounts of Mn^{2+} is almost entirely suppressed if the concentration of pyrophosphate and citrate is sufficiently high. This is shown in Table 9, where the conditions used were the same as for Fig. 3, except that pyrophosphate or phosphate–citrate buffers were employed in place of phosphate. The presence of Mn^{2+} at concentrations above 10^{-5} M caused a lag period in both phosphate–citrate and pyrophosphate buffers before the O_2 uptake started; the lag period with a given amount of Mn^{2+} was greater in the latter two buffers than when phosphate was used. The effect of citrate and pyrophosphate is presumably due to their capacity of forming complex ions with Mn^{2+}. It is not clear why the presence of pyrophosphate and citrate in sufficiently high concentration not only suppresses the activating effect of Mn^{2+} but also increases the lag period.

Effect of pyrophosphate on the activity of the ultrafiltrate. It has been shown that the presence of pyrophosphate strongly inhibits the activating capacity of Mn^{2+} on the peroxidase-catalysed oxidation of IAA by O_2 (Table 9). Therefore, if Mn^{2+} is responsible for a major part of the activity of waxpod bean root ultrafiltrate on the peroxidase-catalysed oxidation of IAA by O_2, then the activity of the ultrafiltrate should be less in pyrophosphate than in phosphate.

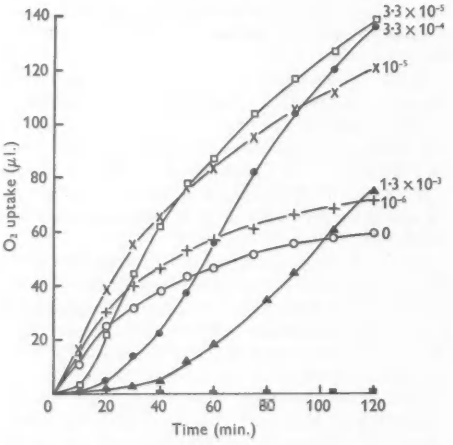

Fig. 3. Effect of variation in Mn^{2+} concentration on the oxidation of IAA by the horseradish peroxidase preparation of P.N. 500. Reaction mixtures consisted of 40 μg. horseradish peroxidase of P.N. 500, 0·067 M phosphate at pH 6 and varying amounts of Mn^{2+}. After equilibration 0·3 ml. 0·05 M IAA was added from the side arm. ○—○, peroxidase; +—+, ×—×, □—□, ●—●, ▲—▲, peroxidase + Mn^{2+}; ■—■, $1{\cdot}3 \times 10^{-3}$ or 10^{-5} M-Mn^{2+}, peroxidase omitted. The number to the right of each curve gives the molarity of Mn^{2+} used.

Table 9. *Effect of pyrophosphate and citrate on the oxidation of IAA by horseradish peroxidase in the presence of* Mn^{2+}

Reaction mixtures as in Fig. 3 except that 0·067 M pyrophosphate, or 0·067 M phosphate plus 0·067 M citrate, were used in place of phosphate at pH 6.

Concentration of Mn^{2+} (M)	O_2 uptake (μl.) Pyrophosphate 60 min.	Pyrophosphate 120 min.	Phosphate–citrate 60 min.	Phosphate–citrate 120 min.
0	70	76	65	71·5
10^{-6}	64	69·5	66	71
10^{-5}	75	79·5	68	79
$3{\cdot}3 \times 10^{-5}$	73·5	82·5	68	87·5
10^{-4}	32·5	71	5	54·5

Table 10. *Effect of pyrophosphate on the activity of ultrafiltrate*

Reaction mixtures consisted of: peroxidase preparation equivalent to 0·02 E.U. 0·067 M pyrophosphate or 0·067 M phosphate at pH 6 and where present ultrafiltrate. After equilibration 0·3 ml. 0·05 M IAA was added from the side arm. The O_2 uptakes are corrected for the blank O_2 uptakes in mixtures from which peroxidase was omitted. Two separate batches of ultrafiltrate were examined (*A* and *B*).

	Uptake of O_2 (μl. in 90 min.) Phosphate	Pyrophosphate
Horseradish (P.N. 1040)	40	48
Horseradish (P.N. 1040) + 0·4 ml. ultrafiltrate *A*	127·5	123
Horseradish (P.N. 1040) + 0·8 ml. ultrafiltrate *A*	199·5	176·5
Horseradish (P.N. 500)	62·5	68·5
Horseradish (P.N. 500) + 0·4 ml. ultrafiltrate *B*	175·5	161

Table 11. *Oxygen uptake and carbon dioxide formation during the oxidation of IAA by peroxidase*

Reaction mixtures consisted of: horseradish peroxidase of R.N. 1040 and where present 1 ml. waxpod bean root ultrafiltrate in 0·067 M phosphate at pH 6. IAA was added from the side arm. The duration of the experiments was 4 hr. The values given are corrected for the blank O_2 uptakes and CO_2 outputs with the systems IAA alone, IAA + ultrafiltrate, and ultrafiltrate + peroxidase. These blanks were very small, the largest being 3 μl. O_2 uptake with the system 10 μmoles IAA + ultrafiltrate.

		IAA (μmoles)					
		10		5		2·5	
Peroxidase (μg.)	Ultrafiltrate	O_2	CO_2	O_2	CO_2	O_2	CO_2
		μmoles					
100	—	4·8	4·7	3·3	3·7	2·3	2·7
300	—	5·5	5·5	4·2	4·1	2·4	2·5
100	1 ml.	7·2	7·7	4·1	4·1	2·3	2·0

The results with two different batches of ultrafiltrate are shown in Table 10. The O_2 uptakes obtained in the presence of pyrophosphate are only slightly less than those obtained in phosphate, which suggests that Mn^{2+} is only responsible for a small part of the activity of the ultrafiltrate. However, there is some doubt concerning the validity of this suggestion since, when amounts of ultrafiltrate in excess of about 0·8 ml. were tested they often caused a lag period in the presence of pyrophosphate and the subsequent rate of O_2 uptake was sometimes less than with smaller amounts of ultrafiltrate.

The course of the oxidation. A study has been made of the O_2 uptake and CO_2 formation when horseradish peroxidase and horseradish peroxidase plus waxpod bean root ultrafiltrate oxidize IAA. The results (Table 11) suggest that, under the conditions used, the oxidation of IAA proceeds more readily to completion with low concentrations of IAA. Thus, with 100 μg. peroxidase the final O_2 uptakes were 0·92 mole O_2/mole IAA when 2·5 μmoles IAA were present, but only 0·48 mole O_2/mole IAA with 10 μmoles IAA. Increasing the amount of peroxidase, or adding ultrafiltrate, increased the final O_2 uptake with the larger amounts of IAA, but was without effect where 2·5 μmoles IAA were present. Using the reagent of Tang & Bonner (1947), approximate colorimetric estimations of IAA in the final reaction mixtures showed that where the O_2 uptake exceeded 0·9 mole O_2/mole IAA, over 90 % of the IAA had been oxidized. The total amount of CO_2 produced was measured by Warburg's direct method. The values obtained were all in the neighbourhood of 1 mole CO_2/mole O_2 consumed.

DISCUSSION

The results of the present work show that highly purified horseradish peroxidase preparations catalyse the oxidation of IAA by O_2 and that in the presence of certain thermostable factors such as monophenols, aniline, resorcinol, manganese and maleic hydrazide the rate of oxidation is increased. The system in waxpod bean root sap responsible for the oxidation of IAA consists of a thermolabile fraction and a thermostable fraction. The evidence suggests that the thermolabile fraction is a peroxidase, since it can be replaced by highly purified horseradish peroxidase preparations. The thermostable fraction in the bean root system has not been identified but the results suggest that a large part of its activity is due to the presence of certain peroxidase substrates. It is considered that Mn^{2+} alone can be responsible for only a small part of the activity of the thermostable fraction; although under certain conditions low concentrations of Mn^{2+} increase the rate of oxidation of IAA by peroxidase, this effect of Mn^{2+} is small compared with that of the ultrafiltrate, particularly when the concentration of peroxidase is low. Furthermore, the activating effect of Mn^{2+} is strongly inhibited by pyrophosphate, while the activity of the thermostable fraction, when present in small amounts, is only slightly affected. However, anomalous results were obtained using large amounts of the thermostable fraction, since under these conditions an inhibiting effect of pyrophosphate was found. Evidence has been obtained that the oxidation of IAA by O_2 catalysed by the peroxidase systems studied is not dependent on the presence of flavoprotein, and this raises the question of the significance of the flavoprotein present in preparations of IAA oxidase from pea epicotyl (e.g. Galston *et al.* 1953). In contrast to the present work, where it was found that the oxidation of IAA by peroxidase proceeded without a lag period or with only a brief one, Galston *et al.* (1953) found that in darkness a lag period of about 2 hr. was necessary before the oxidation commenced. This lag period could be overcome by exposure of the reaction mixtures to white light. Also, Galston *et al.* (1953) found that the inhibiting effect of catalase on the peroxidase-catalysed oxidation of IAA by O_2 was partially reversed by light. These facts suggest that the effect of light in solutions containing peroxidase and IAA

is to increase the rate of production of peroxide. In preliminary experiments it has been found that the rate of oxidation of IAA by highly purified horseradish peroxidase is increased in the presence of strong daylight. This suggests that the stimulating effect of light on the oxidation of IAA by pea epicotyl preparations may not be entirely due to an effect on flavoprotein components, as has been previously supposed (Galston & Baker, 1949, 1951; Galston *et al.* 1953). It is not likely that light had much influence on the results obtained in the present work, since the reactions were carried out in light of very low intensity (< 1 f.c.) except for a brief exposure to daylight during the addition of materials from the side arm of the reaction flasks. However, this brief exposure to light and the different experimental conditions used in the present work may each be partly responsible for the absence of a long lag period before the oxidation of IAA by peroxidase commenced.

The results suggest that the oxidation of IAA by horseradish peroxidase in the absence and in the presence of the thermostable fraction of waxpod bean root proceeds with the consumption of 1 mole O_2/mole IAA oxidized, and the formation of 1 mole CO_2/mole O_2 consumed. These values are in agreement with the findings of Tang & Bonner (1947) and Wagenknecht & Burris (1950) for the IAA oxidizing systems present in extracts of pea epicotyls and waxpod bean roots.

The mechanism of the oxidation is not clear, but at pH 6·5 or 7, which is close to the optimum for catalase action, catalase inhibits strongly, suggesting that the reaction is accompanied by, and depends on, the formation of H_2O_2. The mechanism of the stimulation of the oxidation by Mn^{2+} or maleic hydrazide is obscure, but it is suggested that the activity of the peroxidase substrates such as monophenols, resorcinol, and aniline, depends on their capacity for reversible oxidation–reduction. Such a mechanism would account for the inhibiting effect of polyphenols, since in general these are more readily oxidized by peroxidase than are monophenols, and they would thus successfully compete for the H_2O_2 formed. It presupposes that polyphenols cannot act as redox carriers in the oxidation of IAA, but a similar situation is known in the case of Mn^{2+} oxidation by peroxidase and H_2O_2 where monophenols but not polyphenols are active as carriers (Kenten & Mann, 1950). The fact that those peroxidase substrates (e.g. *p*-chlorophenol) which increase the activity of peroxidase towards IAA are much less effective at high than at low concentration could be due to inhibition of peroxidase by the high phenol concentration. On the other hand, it is possible that these phenols are less easily oxidized by peroxidase and H_2O_2 than is IAA, and that when such substrates are present in high concentration their oxidation is favoured and they compete with IAA for the available H_2O_2. If this latter possibility is correct, then the activity of substrates such as *p*-chlorophenol at low concentration is due to some function other than that of a redox carrier.

The reaction bears a marked resemblance to the known direct oxidase reactions of peroxidase in which the oxidation of dioxymaleic acid (Swedin & Theorell, 1940), certain other dicarboxylic acids (Kenten & Mann, 1953), and phenylacetaldehyde (Kenten, 1953), by O_2 is catalysed by peroxidase. With the dioxymaleic acid reaction it has been previously supposed that H_2O_2 was produced by an initial autoxidation of the dioxymaleic acid, but Hartree (1953) has demonstrated that 'by rigorous purification of the dioxymaleic acid and buffer solution it is possible to abolish autoxidation virtually completely without affecting the rate of the enzyme-catalysed reaction'. Since neither phenylacetaldehyde nor the dicarboxylic acids studied by Kenten & Mann (1953) undergo significant autoxidation under the conditions in which they are oxidized by peroxidase, it is clear that peroxide must arise from some mechanism other than direct autoxidation of the substrate. It is possible, however, that extremely slow autoxidation of these substrates provides traces of H_2O_2 whereby the reaction is initiated, and further H_2O_2 could arise if the oxidation product, produced by the action of peroxidase, autoxidizes readily with the production of H_2O_2.

It is of interest that those compounds which have been demonstrated to increase the activity of IAA oxidase preparations of pea epicotyl, and pineapple leaf and stem tissue, also increase the rate of oxidation of IAA by highly purified horseradish peroxidase. Thus a variety of monophenols, particularly chlorophenols, 4-methylumbelliferone and maleic hydrazide have been shown to activate the pea epicotyl IAA oxidase (Goldacre *et al.* 1953; Andreae & Andreae, 1953), while Mn^{2+} has a large effect on the pineapple IAA oxidase (Gortner & Kent, 1953) and a comparatively small effect with dialysed pea epicotyl extracts (Wagenknecht & Burris, 1950; Goldacre *et al.* 1953). There is ample evidence suggesting the presence of a thermostable inhibitor of IAA oxidase in plant tissues (Tang & Bonner, 1948; Gortner & Kent, 1953) and with pineapple tissue the results of Gortner & Kent (1953) suggest that this may be a polyphenol. In this connexion the inhibiting effect of pyrogallol on the peroxidase catalysed oxidation of IAA by O_2 suggests that peroxidase may be concerned in the oxidation of IAA by pineapple-tissue extracts. The presence, in extracts of horseradish root, of peroxidase substrates which inhibit the reaction is suggested by the fact that, although such extracts are rich in peroxidase, they will not oxidize IAA unless they are first

treated with a suitable amount of H_2O_2. If too large an amount of H_2O_2 is used the activity of the treated extract is considerably less than that of an extract treated with the optimum amount of H_2O_2. This suggests that either the peroxidase in the extract is partially destroyed by exposure to the high concentration of peroxide (Theorell, 1951) or that active peroxidase substrates (e.g. monophenols) are irreversibly oxidized under these conditions.

Further work is necessary before the physiological significance of the peroxidase-catalysed oxidation of IAA by O_2 can be evaluated. Hill & Hartree (1953) have pointed out some of the difficulties in applying the results obtained with distintegrated tissues to a discussion of the metabolism of intact plants. It is known that peroxidase *in vitro* can catalyse the oxidation of a great many different substances by H_2O_2, and several direct oxidase reactions of peroxidase are known. Also, the range of peroxidase action *in vitro* has been further extended by the demonstration that substances which are not directly oxidized by peroxidase and H_2O_2 can be oxidized when certain peroxidase substrates are present and act as carriers. Peroxidase is widely distributed among the higher plants and presumably plays some part in metabolism, but in spite of our knowledge of the *in vitro* activities of peroxidase the relative significance of these activities *in vivo* is not yet known.

SUMMARY

1. The oxidation of indolyl-3-acetic acid (IAA) by oxygen is catalysed by highly purified horseradish peroxidase preparations. The rate of oxidation is increased in the presence of certain thermostable factors such as monophenols, aniline, resorcinol, manganese and maleic hydrazide. With the exception of manganese and maleic hydrazide, all the active factors are known, or have been shown to be, peroxidase substrates. Other peroxidase substrates, e.g. catechol, hydroquinone, pyrogallol and *p*-phenylenediamine (10^{-4}–10^{-5} M) strongly inhibit the peroxidase-catalysed oxidation of IAA.

2. The IAA oxidase of waxpod bean root sap consists of a thermolabile fraction, which appears to be a peroxidase and which can be replaced by horseradish peroxidase, and a thermostable fraction, the activity of which largely depends on the presence of peroxidase substrates.

3. The oxidation of IAA when catalysed by horseradish peroxidase in the absence or presence of waxpod bean root thermostable fraction proceeds with the consumption of 1 mole oxygen/mole IAA oxidized and the formation of 1 mole carbon dioxide/mole oxygen consumed.

4. Possible mechanisms of the reaction are discussed, and evidence is presented suggesting that the oxidation of IAA by the peroxidase systems studied is not dependent on the presence of flavoprotein.

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Studies on Rhodopsin

8. RETINYLIDENEMETHYLAMINE, AN INDICATOR YELLOW ANALOGUE*

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The action of light on rhodopsin (visual purple) produces a pigment named, by Lythgoe (1937), 'indicator yellow' because it changes colour from a very pale yellow ($\lambda_{max.}$ 365 mμ.) in alkaline solution to a much deeper yellow ($\lambda_{max.}$ 440 mμ.) on addition of acid. (In this paper, the terms indicator yellow, vitamin A and retinene will be used to mean indicator yellow$_1$, vitamin A$_1$ and retinene$_1$.) Collins & Morton (1950) and Collins (1953) have offered evidence that indicator yellow is not merely an artifact (using the word artifact here to mean a secondary product having no place in a cycle occurring under physiological conditions); in their view indicator yellow is more truly a derivative of rhodopsin, and it is the first photodecomposition product detectable in water at room temperature. Proof of the chemical nature of indicator yellow should be relevant to the structure of the rhodopsin chromophore, a fundamental issue which has so far been approached only tentatively.

The visual pigments and their derivatives possess characteristic absorption spectra, but the amounts present in the retina are too small to permit their characterization by ordinary chemical means. Even if sufficient material were obtainable, it is doubtful if chemical investigations would throw much light on the nature of a compound such as rhodopsin or indicator yellow formed from retinene (molecular weight, 284) and a protein (opsin) with a molecular weight of about 40 000. The most promising approach is to prepare a substance spectroscopically similar, but not containing the large protein moiety.

The final product of the action of light on rhodopsin *in vitro* is retinene (vitamin A aldehyde). A close relationship between this and indicator yellow became evident when retinene was found to link with many substances containing amino groups, i.e. amines, amino acids and proteins, to give products spectroscopically similar to indicator yellow (Ball, Collins, Dalvi & Morton, 1949). These retinene-amino compounds clearly resemble the indicator yellow derived from rhodopsin and have been called 'indicator yellow analogues'.

Two possible structures have been put forward for these analogues in an alkaline medium: Ball *et al.* (1949) suggested they might be Schiff's bases (I), formed by the condensation of one molecule of retinene with one amino group of the amine or protein (R.NH$_2$).

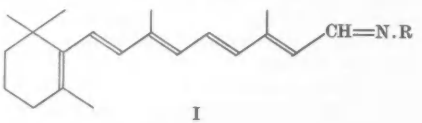
Collins & Morton (1950) tentatively proposed, for compounds other than those formed from aromatic amines, a tertiary amine structure in which two molecules of retinene joined with one amino group. They emphasized that proof of these suggested structures was lacking, as the substances were known only in solution.

The present paper describes the formation from retinene and methylamine of a crystalline indicator yellow analogue, which is shown to be a Schiff's base (I, R = CH$_3$). This structure could not be accepted without careful scrutiny because of the difficulty of explaining the shift of $\lambda_{max.}$ in acid solution. Evidence is now advanced that the displacement can be accounted for by the addition of a proton to the nitrogen atom to form a substituted ammonium salt (II, R = CH$_3$, R′ = H).

RESULTS

Retinene was condensed with methylamine and the product obtained as yellow crystals showing in solution the colour changes characteristic of natural indicator yellow and its synthetic analogues. Spectroscopic details are given in Table 1, and absorption curves in Figs. 1–3. Analytical results were: C, 84·7; H, 10·4; N, 4·9 %. Mol.wt. 290. Values calculated for the two previously proposed structures are: Schiff's base $C_{21}H_{31}N$ (Ball *et al.* 1949) C, 84·9; H, 10·4; N, 4·7 %. Mol.wt. 297. Tertiary amine $C_{41}H_{57}N$ (Collins & Morton, 1950) C, 87·4; H, 10·1; N, 2·5 %. Mol.wt. 563.

* No. 7 of this series: Collins, Green & Morton (1954).



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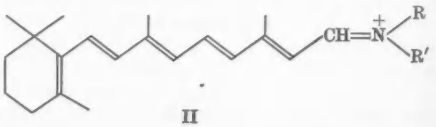

II

These results agree with the suggestion that the indicator yellow analogue formed from retinene and methylamine is a Schiff's base (I, R = CH_3), 3:7-dimethyl-9-(2:6:6-trimethyl*cyclo*hex-1-en-1-yl)-nona-2:4:6:8-tetraenylidenemethylamine. We have given it the trivial name of retinylidenemethylamine. (In our proposed nomenclature for retinene derivatives—see p. 126—retinylidene is the name given to the $C_{19}H_{27}CH=$ radical in I.)

This structure contains the same chromophoric group as retinene oxime (I, R = OH), which should therefore have an absorption spectrum very similar to that of the indicator yellow analogue, if the Schiff's base structure is correct. Retinene oxime has been prepared by Farrar, Hamlet, Henbest & Jones (1952) and in greater purity by Wald & Brown (1953). Its molecular extinction coefficient has been reported only in polar solvents. Retinene oxime was therefore again prepared in order to compare its absorption spectrum and antimony trichloride colour reaction with those of retinylidenemethylamine. Details are given in Table 2. $\epsilon_{max.}$ in ethanol agrees well with that obtained by Wald & Brown (1953). Fig. 2 shows the absorption spectra of retinene oxime and retinylidenemethylamine in light petroleum, a convenient non-polar solvent for obtaining a just comparison. The nearly identical curves support the suggested Schiff's base structure of the indicator yellow analogue.

The antimony trichloride (Carr-Price) colour reactions of retinene oxime and retinylidenemethylamine differ somewhat in the position and intensity of the maximum but share features not found in the Carr-Price chromogens of other vitamin A derivatives. (1) The two compounds have colour-test maxima in the same spectral region, and at much shorter wavelengths (480 and 505 mμ.) than would be expected for compounds with six conjugated double bonds (cf. retinene, 664 mμ.; anhydrovitamin A, 620 mμ.). (2) The molecular extinction coefficients in the colour test are of the same order and are unexpectedly low (< 50 000) (cf. retinene, $\epsilon_{max.}$, 108 000 (Hubbard, Gregerman & Wald, 1953); anhydrovitamin A, $\epsilon_{max.}$, 145 000 (Cama, Collins & Morton, 1951)). (3) The colour-test absorption curves are broad with rounded peaks and half band-widths (distance in mμ. separating ϵ values at $\epsilon_{max.}/2$) of approx. 110 mμ. The half-width for anhydrovitamin A in antimony trichloride is 56 mμ. (calc. from Cama *et al.* 1951). (4) The chromogens are unusually stable. With retinyl-

Table 1. *Spectroscopic properties of retinylidenemethylamine*

Solvent	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$
Light petroleum	355	57 400
*Water (0·5 M methylamine)	365	49 300
*Water (0·1 N-HCl)	438	41 000
$CHCl_3$	366	53 200
$CHCl_3$ + HCl	466	50 500
$SbCl_3$ colour reaction	505	47 000

* Digitonin used as dispersing agent.

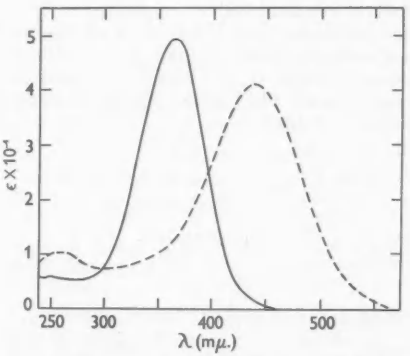

Fig. 1. Absorption spectrum of retinylidenemethylamine in water + methylamine, ——; in water + HCl, – – –. (Dispersing agent–digitonin.)

Table 2. *Spectroscopic properties of retinene oxime*

Solvent	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$
Light petroleum	356	58 300
Ethanol	358	60 000
$SbCl_3$ colour reaction	480	40 000

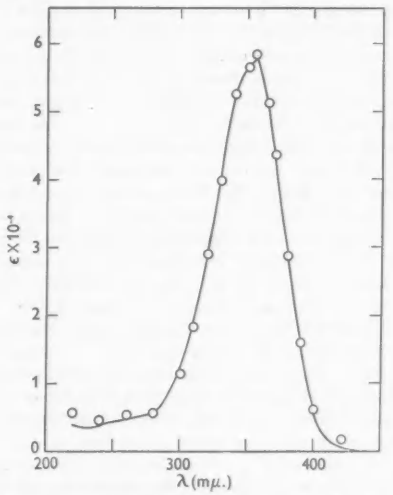

Fig. 2. Absorption spectrum in light petroleum of retinylidenemethylamine, ——; points experimentally determined for retinene oxime, ○.

idenemethylamine, the red colour fades very slowly and falls to half its original intensity after about a day; $\lambda_{max.}$ moves from its original position at 505 mμ. to 495 mμ. within a few min., but on standing for several hr. it moves back to 508 mμ. With retinene oxime the colour fades more quickly, but even so persists for several hr.

Stability of retinylidenemethylamine

Retinylidenemethylamine is extremely unstable. It is rapidly hydrolysed by mere traces of ethanol or water over a wide pH range, unless a great excess of methylamine is present (see Morton & Pitt, 1955) and it is readily attacked by atmospheric oxygen. If a solution in light petroleum is left exposed to the air for 30 min. at room temperature, an insoluble red breakdown product is deposited.

Owing to its instability, retinylidenemethylamine is difficult to purify by adsorption chromatography. It is broken down to retinene by strong adsorbents such as alumina, and although it is reasonably stable on weak adsorbents, such as calcium carbonate, these are not strong enough to separate it from accompanying impurities.

The preparation of pure retinylidenemethylamine is therefore a difficult task. The best method was found to be repeated crystallization from light petroleum at $-78°$ in an atmosphere of nitrogen with exclusion of moisture. Without these precautions the product was impure and gave incorrect analytical results.

The 'acid' form of retinylidenemethylamine

On passing dry hydrogen chloride through a light petroleum solution of retinylidenemethylamine, a red material is precipitated (m.p. (decomp.) about 140°) which, when dissolved in chloroform, gives the absorption curve characteristic of the 'acid' form of retinylidenemethylamine. This is to be expected if a substituted ammonium salt is formed, as it will be insoluble in a non-polar solvent. Attempts to purify this crude product by crystallization failed, and analyses showed the presence of a variable excess of chloride and oxygen, presumably as a result of side reactions. The simplest explanation is that, in acid, retinylidenemethylamine forms a substituted ammonium salt (II, $R=CH_3$ $R'=H$) as a typical Schiff's base. A possible alternative is a doubled molecule, the increase of $\lambda_{max.}$ being accounted for by the elongation of the chain of conjugated double bonds. Two lines of evidence, however, indicate that the simpler explanation is almost certainly correct.

The addition of a proton to retinylidenemethylamine introduces a positive charge into the nitrogen atom. This charge (which implies a local deficiency of electrons) could formally be localized at many positions along the polyene chain. At least seven distinct resonance forms can be written for the retinylidenemethylammonium ion compared with one main form for retinylidenemethylamine, resonance forms with low probability being neglected. Such increase in the degree of resonance would contribute to a shift in $\lambda_{max.}$ towards the visible.

So far as we know, it is not possible to find in the literature experimental evidence of such a big bathochromic shift associated unambiguously with a change of a —CH=N— grouping to —CH=NH^+—. To examine the validity of this idea, it is necessary to see if other similar compounds behave as retinylidenemethylamine.

Schiff's bases were formed in solution from methylamine with benzaldehyde, cinnamaldehyde, *β-apo*-12′-carotenal and *β-apo*-8′-carotenal. These *apo*carotenals (which were kindly provided by Mr E. R. Redfearn and Dr J. Glover) are aldehydes with polyene chains longer than that of retinene. On formation of the Schiff's bases in ethanolic solution by a method substantially that of Ball *et al.* (1949), a rise in extinction was observed. No marked change of $\lambda_{max.}$ was seen with benzaldehyde, but cinnamaldehyde showed a hypsochromic shift which was much more marked with the *apo*carotenals (Table 3). Similar effects are seen when retinene ($\lambda_{max.}$ (ethanol), 383 mμ.; $\epsilon_{max.}$, 42 900 (Wald & Brown, 1953)) is converted into retinylidenemethylamine ($\lambda_{max.}$ (ethanol), 362 mμ.; $\epsilon_{max.}$, 55 800). The rise in extinction in each case shows that the Schiff's base of the aldehyde has been formed. Although benzaldehyde did not show the hypsochromic shift accompanying Schiff's base formation in the other aldehydes, such a shift is not always found with short conjugated chains. Indeed, when crotonaldehyde is converted into crotonylidene butylamine, although the extinction rises, $\lambda_{max.}$ moves slightly to longer wavelengths (Hausser, Kuhn, Smakula & Hoffer, 1935; Barany, Braude & Pianka, 1949).

On acidification, the solutions of all the Schiff's bases showed a bathochromic shift. Absorption maxima of these compounds are summarized and compared with the corresponding retinene derivatives in Table 3.

Table 3. $\lambda_{max.}$ *of methylamine derivatives of aldehydes*

Solvent, ethanol.

Aldehyde	$\lambda_{max.}$ (mμ.)	$\lambda_{max.}$ of Schiff's base (mμ.)	
		In alkali	In acid
Retinene	383	362	439
Benzaldehyde	246	245	273
Cinnamaldehyde	286	282	324
β-apo-12′-Carotenal	421	403	486
β-apo-8′-Carotenal	459	444	520

On acidifying, there was a fall in extinction of the *apo*carotenylidenemethylamine solutions similar to that seen with retinylidenemethylamine, but the extinction of the cinnamylidenemethylamine solution rose. Benzylidenemethylamine decomposed at an appreciable rate in acid, so that no accurate quantitative measurements were made. An increase in the degree of resonance (postulated as an explanation of the bathochromic shift) would be expected to cause a rise in extinction, as is seen with cinnamylidenemethylamine. It is somewhat unexpected that this is not found with the aliphatic Schiff's bases containing longer polyene chains, but the *apo*carotenylidenemethylamines parallel retinylidenemethylamine closely in this respect.

From these spectroscopic observations, it appears that the 'acid shift' found in retinylidenemethylamine is probably due to the conversion of the nitrogen atom to the quaternary state. To confirm this, another retinylideneammonium derivative of structure II was made.

Alkyl iodides are known to unite with Schiff's bases to give substituted ammonium iodides (Decker & Becker, 1913). Methyl iodide was allowed to react with retinylidenemethylamine to form a product insoluble in light petroleum, but which dissolved in chloroform to give a broad curve (half-width 102 mμ.) with $\lambda_{max.}$ 485 mμ. (Fig. 3). This is presumably retinylidenedimethylammonium iodide (iodide of II, $R = R' = CH_3$). The material was stable in anhydrous ethanol and showed $\lambda_{max.}$ 455 mμ., but on dilution with water decomposed to give retinene. It also decomposed on making the ethanolic solution slightly alkaline (0·001 M-KOH).

Addition of antimony trichloride in chloroform gave a broad absorption band (half-width about 120 mμ.) with a maximum at 505 mμ., changing to 497 mμ. within a few minutes. The colour was relatively stable; after 7 hr., the intensity had dropped only to 73 % of that originally developed, but $\lambda_{max.}$ had moved to 515 mμ. No other peak appeared at longer or shorter wavelengths.

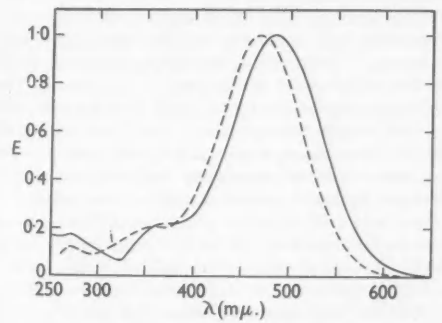

Fig. 3. Absorption spectra in chloroform: retinylidenemethylammonium chloride, – – –; retinylidenedimethylammonium iodide, ——. Shown as $E_{max.} = 1·0$ (absolute value of $E_{max.}$ not known for retinylidenedimethylammonium iodide, but within 5 % of that of retinylidenemethylammonium chloride).

Accurate determinations of the molecular extinction coefficients were not made owing to difficulty in purifying the material, but fairly reliable estimations of $\epsilon_{max.}$ in chloroform and of $\epsilon_{max.}$ of the antimony trichloride colour reaction showed them to be within 5 % of those found with the acid form of retinylidenemethylamine.

The absorption spectrum in chloroform of retinylidenedimethylammonium iodide bears a strong resemblance to that of the acid form of retinylidenemethylamine (see Fig. 3). Both have relatively broad bands of approximately the same intensity, with an inflexion around 350–380 mμ. The difference of 19 mμ. (466 – 485) in $\lambda_{max.}$ is presumably accounted for by the different substituents on the quaternary nitrogen atom; such an effect is commonly found among dyes (for a number of examples, see Dewar, 1949).

The strong resemblances in absorption spectrum and antimony trichloride colour reaction of retinylidenedimethylammonium iodide and the acid form of retinylidenemethylamine confirm that the shift with pH of $\lambda_{max.}$ of retinylidenemethylamine is due to the conversion of the nitrogen atom to the quaternary state.

Natural indicator yellow, derived directly from rhodopsin, therefore has structure I (R = opsin) in alkali, and in acid, structure II (R = opsin, R′ = H).

Retinylidenemethylamine in concentrated sulphuric acid

Unstable coloured products, including those similar to their Carr-Price chromogens, are formed by vitamin A and retinene when acted upon by concentrated sulphuric acid (Ball & Morton, 1949). As antimony trichloride gives an unusual colour reaction with retinylidenemethylamine, it is of interest to know the effect of concentrated acid.

On dissolving retinylidenemethylamine in concentrated sulphuric acid (98 %, w/w) a red-purple colour appeared ($\lambda_{max.}$ 515–520 mμ.). This faded very rapidly at room temperature, leaving a fairly stable peak about 430–435 mμ., with an extinction of about 0·3 of that initially observed at 520 mμ. No other peaks were found. Owing to the rapid fading of the 515–520 mμ. band, its extinction was not measured accurately, but it did not differ from that of the Carr-Price chromogen by more than 15 %.

Vitamin A and retinene in concentrated sulphuric acid give a number of peaks between 440 and 650 mμ. (Ball & Morton, 1949). Retinylidenemethylamine in concentrated sulphuric acid, as in

antimony trichloride solution, seems to be inhibited from forming chromogens with maxima on the long-wavelength side of 520 mμ.

MATERIALS AND METHODS

Antimony trichloride colour reaction. To a solution of the substance in $CHCl_3$ was added a saturated solution of anhydrous $SbCl_3$ in ethanol-free $CHCl_3$ containing approx. 1% (v/v) acetic anhydride.

Absorption spectra were determined with a photoelectric spectrophotometer, except for some observations on the H_2SO_4 chromogen, made using a Hilger-Nutting visual instrument.

Analyses were by Weiler and Strauss, Oxford, and in some cases by the Department of Organic Chemistry, University of Liverpool, where the molecular-weight determination was made (Rast, camphene).

All work involving vitamin A, retinene or derivatives was carried out in red light or in very dim artificial light to avoid isomerization or photodestruction.

Light petroleum. The commercial product (b.p. 40–60°) was used, except for absorption spectra, which were determined in light petroleum (b.p. 60–80°) free from aromatic hydrocarbons.

Chloroform. For the alkaline spectrum of retinylidenemethylamine, $CHCl_3$ was first exposed to NH_3 vapour to remove any traces of HCl. For the acid spectrum, a little anhydrous HCl was bubbled through the $CHCl_3$.

Aqueous solutions of retinylidenemethylamine. Digitonin (1%, w/v) was used as dispersing agent. Solid retinylidenemethylamine was dissolved in a small volume (<0·1 ml.) of ethanol containing excess methylamine to prevent hydrolysis (see Morton & Pitt, 1955) and the digitonin solution added. For the alkaline spectrum, the digitonin solution contained methylamine (0·5M) and for the acid spectrum, HCl (0·1N).

Retinene. This was prepared from synthetic *all-trans*-vitamin A (Hoffman-La Roche) by oxidation with MnO_2 (Ball, Goodwin & Morton, 1948). The crystalline material was used.

Retinylidenemethylamine. Retinene (1 g.) was dissolved in light petroleum (50 ml.) and methylamine bubbled through. Anhydrous Na_2SO_4 (5 g.) was added and the mixture left at room temperature. After 1 hr. the $SbCl_3$ colour reaction of a small portion was examined. A band at 500 mμ. with no sign of a subsidiary band at 664 mμ. (due to retinene) confirmed that the reaction was complete. The solution was filtered through a sintered-glass funnel (porosity 4) and the filtrate dried at reduced pressure and room temperature. The residue crystallized at −78° from light petroleum as yellow needles. Repeated recrystallizations raised the m.p. to 76° (uncorr.).

Retinene oxime. Hydroxylamine hydrochloride (20 g.) was dissolved in water (30 ml.) and a 60% (w/v) solution of KOH in water added to bring the pH to over 10·5. The mixture was poured on to a solution of retinene (1·7 g.) in ethanol (50 ml.). After 10 min., $\lambda_{max.}$ of the solution had changed to 358 mμ., indicating substantially complete conversion into the oxime. The reaction mixture was diluted with water (500 ml.) and extracted with ether. The extract was evaporated to dryness and the crude oxime recrystallized from methanol. M.p. 139–142° (uncorr.).

Methylamine derivatives of other aldehydes. To an ethanolic solution of the aldehyde (diluted to give a convenient reading in the spectrophotometer) were added 3 drops of a solution of methylamine (33% w/v) in ethanol. After 1 hr., the absorption curve of the Schiff's base was determined. Conc. HCl (4 drops) was added and the absorption curve examined again.

Retinylidenedimethylammonium iodide. Methyl iodide (5 ml.) was poured on to solid retinylidenemethylamine (102 mg.) and left at room temperature for 40 min. The excess methyl iodide was removed by evaporation under reduced pressure at room temperature. The red residue was washed with light petroleum to remove any soluble material. Attempts to purify it by crystallization failed. The crude product had m.p. 140–145° (decomp.). (Found: C, 57·2; H, 7·3; N, 3·2; I, 31·7. $C_{22}H_{34}NI$ requires C, 60·1; H, 7·8; N, 3·2; I, 28·9%.)

DISCUSSION

Nomenclature

Many workers are satisfied with the name vitamin A_1 for $C_{20}H_{29}OH$ and vitamin A_2 for $C_{20}H_{27}OH$ and indeed there is much to be said for retaining these names in certain contexts. The same considerations apply to retinene$_1$ and retinene$_2$. The name 'axerophthol' for vitamin A_1, suggested some years ago, has not won general acceptance.

It is difficult, as the subject advances, to resist indefinitely the need for a somewhat more systematic nomenclature for purely chemical designations, however appropriate it may be to retain the older names in general nutritional and biochemical contexts.

Wald (1935) chose the name retinene because he regarded the compound as a carotenoid, but when it was identified as vitamin A aldehyde (Morton & Goodwin, 1944) the suggestion was made that 'retinaldehyde' or 'retinal' would be more suitable. Logically, vitamin A alcohol would be retinol; vitamin A_2, 3-dehydroretinol; and retinene$_2$, 3-dehydroretinal.

The problem of nomenclature is brought to a head with the case of a Schiff's base made from retinene$_1$. 'Retinene$_1$-methylimine' (Collins, 1953) is obviously only a temporary expedient. The minimum departure from current practice would be to use retinal for retinene$_1$. On this basis the Schiff's base becomes retinylidenemethylamine and indicator yellow analogues retinylideneamines. Natural indicator yellow$_1$ would be *N*-retinylidene-opsin, and acid indicator yellow would be a salt of the ammonium form of retinylidene-opsin. Similarly, indicator yellow$_2$ would be *N*-3-dehydroretinylidene-opsin. A refinement of this nomenclature to distinguish between different forms of indicator yellow is suggested by Morton & Pitt (1955).

This usage is serviceable in the present context and goes far to meet the objections of Wald (1953) to the trivial names (such as indicator yellow) now in use for visual pigments.

Antimony trichloride colour reactions

There is no certainty concerning the precise structure of the chromogens produced by the addition to polyene compounds of an antimony trichloride solution, and there is evidence that antimony trichloride itself is not the active substance (Brüggeman, Krauss & Tiews, 1952). It is, however, generally agreed that the chromogens are ionized forms of the polyenes (Meunier, 1942).

Those recorded in this paper as derived from retinylidenemethylamine, retinylidenedimethylammonium iodide, and retinene oxime differ markedly from those given by other vitamin A derivatives but resemble one another closely. It seems probable that all three Carr-Price chromogens are of similar type. The coloured compound formed by retinylidenemethylamine in concentrated sulphuric acid bears a superficial resemblance to them.

Whatever the nature of these coloured compounds may be, it is obvious that the introduction of an azomethine grouping into a polyene has a very great influence on the manner in which the molecule can ionize.

Artificial chromogens and rhodopsin

The cardinal difficulty in approaching the structure of rhodopsin is to account for the displacement of $\lambda_{max.}$ of retinene from 385 to 500 mμ. on uniting with the protein opsin. In the past, attempts have been made to overcome this by postulating that two conjugated molecules of retinene were involved, but Hubbard (1954) has shown that the chromophore of rhodopsin contains only one molecule of retinene.

It has been suggested (e.g. Wald, 1948) that the rhodopsin chromophore is some ionized form of retinene, but how this could be formulated so as to account for the absorption band with a maximum at 500 mμ. has remained puzzling. The antimony trichloride and sulphuric acid colour reactions reported here with retinylideneamine derivatives are the nearest approach to reproducing the chromophore. They have maxima near 500 mμ. and, in the case of Carr-Price chromogens, they give broad absorption curves similar to that of rhodopsin: the half-bandwidth of rhodopsin is 100 mμ. (Collins, Love & Morton, 1952), of retinylidenemethylamine and antimony trichloride about 110 mμ. (As the sulphuric acid colour produced was unstable, its bandwidth was not determined.) These chromogens have molecular extinction coefficients of the same order as that of the rhodopsin chromophore, and they are formed from retinylideneamino compounds which are known to be produced in the retina from the carbon–nitrogen linkage present in rhodopsin (Collins, 1953). Further, although most derivatives of vitamin A when ionized give coloured products with $\lambda_{max.}$ at wavelengths near or above 600 mμ., retinylideneamine derivatives cannot be induced, even in strongly ionizing media, to give absorption peaks at wavelengths much longer than 500 mμ.

These artificial chromogens are not, however, photosensitive, and their production under such grossly unphysiological conditions may have no relevance to the changes undergone by retinene on uniting with opsin in the eye. Nevertheless, this work brings out potentialities of retinylideneamino compounds which deserve consideration in future investigations on the structure of rhodopsin.

It will be noticed (Fig. 3) that both the retinylideneammonium salts in chloroform solution show a marked inflexion around 350–380 mμ., and that rhodopsin also shows a small peak (β-band) in this region. The rhodopsin band may be a *cis*-peak (Hubbard & Wald, 1952), but it is interesting that an inflexion is found here associated with an apparently *all-trans*-retinylideneammonium chromophore.

SUMMARY

1. A crystalline compound showing the spectroscopic characteristics of indicator yellow has been prepared from retinene and methylamine.

2. Analytical results show this to be a Schiff's base, to which the trivial name of retinylidenemethylamine has been given. (The $C_{19}H_{27}CH=$ radical has been named retinylidene—this nomenclature is discussed.)

3. The spectroscopic properties and antimony trichloride colour reactions of retinene oxime are reported, and confirm the Schiff's base structure of the indicator yellow analogue.

4. Corresponding Schiff's bases have been prepared in solution from methylamine and other aldehydes and show similar shifts of $\lambda_{max.}$ in acid.

5. Retinylidenemethylamine reacts with methyl iodide to give retinylidenedimethylammonium iodide, which has $\lambda_{max.}$ (chloroform) 485 mμ.

6. These results show that the bathochromic shift of retinylidenemethylamine in acid is due to the formation of a retinylideneammonium salt.

7. Indicator yellow derived directly from rhodopsin is therefore *N*-retinylidene-opsin and 'acid indicator yellow' the corresponding ammonium salt.

8. Unusual chromogens are produced from retinylideneamino compounds by the action of antimony trichloride and concentrated sulphuric acid. Their resemblance to the rhodopsin chromophore is discussed.

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Studies on Rhodopsin

9. pH AND THE HYDROLYSIS OF INDICATOR YELLOW

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Lythgoe (1937) showed that on exposure to light, rhodopsin (visual purple) solutions break down to give the pigment 'indicator yellow', which has $\lambda_{max.}$ about 365 mμ. in alkali ('alkaline' indicator yellow) and about 440 mμ. in acid ('acid' indicator yellow) (Collins & Morton, 1950).

The pH affects not only the spectrum but also the stability of indicator yellow. Lythgoe (1937) found his preparations to be stable above pH 7 in the alkaline form and fairly stable in the acid form at pH 3·3. Between pH 4·0 and 6·1, however, the acid form decomposed to give what Bliss (1948) later identified as retinene (vitamin A aldehyde). To explain this behaviour Lythgoe postulated two forms of acid indicator yellow—one stable (existing at pH 3·3), the other unstable (existing between pH 4·0 and 6·1). Wald (1938), Bliss (1948) and Collins & Morton (1950) have reported acid indicator yellow as unstable, but they all studied indicator yellow solutions near pH 4·0, i.e. they were dealing with the unstable form. Lythgoe's stable acid indicator yellow has largely been overlooked.

Substances behaving spectroscopically as indicator yellow, and known as indicator yellow analogues, have been made by the interaction of retinene and amines (Ball, Collins, Dalvi & Morton, 1949). These are Schiff's bases—retinylideneamines—which in acid solution form retinylideneammonium salts (using the nomenclature of Pitt, Collins, Morton & Stok, 1955). It has been observed in this laboratory (F. D. Collins, unpublished) that, in fairly strongly acid solutions, these indicator yellow analogues are stable, in the sense that they do not hydrolyse to retinene. The availability of retinylidenemethylamine (a crystalline indicator yellow analogue; Pitt *et al.* 1955) allows its stability to be studied in aqueous solution without interference caused by other substances inevitably present in solutions of natural indicator yellow obtained from eyes.

Such a study is reported in this paper and, based on the knowledge thus obtained, further investigations have been carried out of the relationship between indicator yellow and rhodopsin.

EXPERIMENTAL

Absorption spectra were determined by means of a photoelectric spectrophotometer.

Retinylidenemethylamine. This was prepared by the method of Pitt *et al.* (1955). The crystalline material was used.

Aqueous solutions of retinylidenemethylamine. These were made using Tween 80 (polyoxyethylene derivative of sorbitan mono-oleate, Honeywill and Stein, Ltd., St James' Square, London, S.W. 1) by the technique of Bliss (1951) and were of concentrations suitable for reading in the spectrophotometer (approx. M $\times 10^{-5}$).

In this study, Tween 80 is more convenient than digitonin, which is accepted as the best aid to the extraction of rhodopsin and thence of indicator yellow. Digitonin slowly precipitates from aqueous solutions (particularly when acid), thereby causing turbidity and interference with spectrophotometric measurements. The substances with which this investigation is concerned have their spectra in Tween 80 solutions moved to shorter wavelengths as compared with those found in digitonin solutions. The differences are summarized in Table 1. (This shift is responsible for one of the apparent differences between indicator yellow and retinylidenemethylamine reported by Collins (1953) because he studied indicator yellow in digitonin solution, and retinylidenemethylamine in Tween 80.) Retinylidenemethylamine solutions of different pH values were made using: pH values 1 and 2, HCl; 3, acetic acid; 3·7 and 4·8 acetate buffer; 5·7, phthalate buffer; 6·3, 6·6 and 7·7, phosphate buffer; 8·6, borate buffer; 13, KOH. The pH of the resulting solution was checked at the end of the experiment, using a Lovibond comparator, except for those of pH values 1, 2 and 13, which were made using standardized acid and alkali.

Rhodopsin. Solutions were prepared from ox retinas by a slight modification of the method of Collins, Love & Morton (1952). After the alum treatment, the rods were washed with 0·67 M phosphate buffer of pH 6·3 (Clark, 1928), mixed with anhydrous Na_2SO_4, extracted twice with light petroleum (b.p. 40–60°) and washed with water until free from sulphate. The rhodopsin was then extracted with a 1% (w/v) solution of digitonin in 0·67 M phosphate buffer (pH 6·3).

Acid destruction of rhodopsin. The absorption spectrum of the rhodopsin solution (2 ml.) was recorded. HCl (0·03 ml., 10 N) was added in the dark, and the absorption spectrum examined rapidly. KOH (0·05 ml., 30%, w/v) and 0·5 ml. of approx. 1·5 M methylamine containing excess KOH were added. After 5 min., the mixture was reacidified with 0·1 ml. 10 N-HCl and the absorption curve redetermined.

RESULTS

pH *and hydrolysis of retinylidenemethylamine*

Aqueous solutions of retinylidenemethylamine were made at different pH values. The absorption spectra were recorded immediately after mixing and observations continued so that any change could be followed. In most cases $\lambda_{max.}$ moved to 380–385 mμ., showing that retinylidenemethylamine was being hydrolysed to retinene. The time taken for $\lambda_{max.}$ to change to 380–385 mμ. was noted. Results are shown in Table 2.

Table 1. *Effect of Tween* 80 *and digitonin on* $\lambda_{max.}$ *of aqueous dispersions*

Compound	$\lambda_{max.}$ (mμ.) In digitonin	$\lambda_{max.}$ (mμ.) In Tween 80
Retinene*	389	383
Retinylidenemethylamine (in alkali)	365	362
Retinylidenemethylamine (in acid)	438	430

* $\lambda_{max.}$ of retinene in aqueous solutions is not changed in acid or alkali.

At pH 1·0 no hydrolysis of retinylidenemethylammonium chloride occurred. The absorption curve observed immediately on mixing, and that after standing at room temperature for 18 hr. are shown in Fig. 1. After 18 hr. $\lambda_{max.}$ was still at 430 mμ., but there was a general fall in extinction

Table 2. *Hydrolysis of retinylidenemethylamine in aqueous solution*

Tween 80 used as dispersing agent.

pH	$\lambda_{max.}$ (mμ.) immediately after mixing	$\lambda_{max.}$ moved to 380–385 mμ. after
1·0	430	No change
2·0	430	30 hr.
3·0	430	Approx. 10 hr.
3·7	428	90 min.
4·8	415	35 min.
5·7	397	10 min.
6·6	367	28 min.
7·7	363	75 min.
8·6	363	100 min.
13	363	110 min.
In the presence of 0·2 M methylamine		
3·7	424	90 min.
6·3	366	60 min.
8·6	362	No change
In the presence of 4% (w/v) aqueous formaldehyde		
8·6	370	9 min.

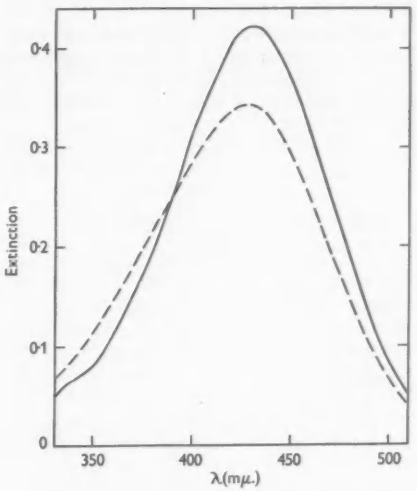

Fig. 1. Absorption spectrum of retinylidenemethylamine in 0·1 N-HCl: on mixing, ——; after 18 hr., – – –. (Dispersing agent—Tween 80.)

at wavelengths above 390 mμ. and a rise at shorter wavelengths. This is presumably due to non-hydrolytic destruction of retinylidenemethylammonium chloride, such as commonly occurs with derivatives of vitamin A in polar solvents at room temperature, particularly when exposed to air. Also acid tends to isomerize carotenoids (Zechmeister, 1944) and may well isomerize retinylidenemethylammonium chloride in these conditions, causing a drop in extinction. There is, however, no indication of any measurable hydrolysis to retinene, which would be marked by a peak or rise in extinction at 383 mμ.

At pH values of 2·0 and 3·7, the retinylidenemethylammonium salt initially formed hydrolyses to retinene—being less stable at the higher pH. Fig. 2 shows the hydrolysis at pH 3·7. There is an isosbestic point at 400 mμ., indicating that the effective absorption is due to only two substances.

At pH values 4·8 and 5·7, the initial $\lambda_{max.}$ lies between the maxima of retinene and the retinylidenemethylammonium salt, and very rapidly changes over to give the curve characteristic of retinene. The same changes are seen as at pH 3·7, but are proceeding faster so that the retinylidenemethylammonium salt has partially hydrolysed before it can be examined in the spectrophotometer.

The solution at pH 6·6 shows a maximum between those of retinylidenemethylamine (the uncharged Schiff's base) and retinene. At pH values above this, retinylidenemethylamine is more stable and hydrolyses more slowly to retinene. A figure showing the hydrolysis of retinylidenemethylamine has been given by Collins (1953).

When this behaviour of retinylidenemethylamine is compared with that of indicator yellow formed from rhodopsin solution (Lythgoe, 1937), it is seen that the stability of the acid indicator yellow

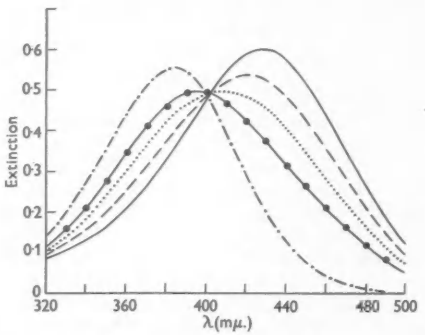

Fig. 2. Absorption spectrum of retinylidenemethylamine in aqueous solution at pH 3·7: on mixing, ——; after 12 min., – – –; after 20 min., · · · ·; after 35 min., •—•; after 75 min., –·–·–. (Dispersing agent—Tween 80.)

reported by Lythgoe is paralleled by the resistance of retinylidenemethylammonium chloride to hydrolysis at pH 1. Similarly, the hydrolysis of acid indicator yellow in a less acid pH range agrees with the behaviour of the analogue. However, hydrolysis of retinylidenemethylamine still occurs in alkaline solution (previously reported by Collins, 1953), whereas in solutions prepared from the eye, alkaline indicator yellow is stable.

This difference in behaviour can be explained by considering the hydrolysis of retinylideneamines to retinene and the free amine:

$$C_{19}H_{27}CH{=}N.R + H_2O \rightleftharpoons C_{19}H_{27}CHO + R.NH_2. \quad (1)$$

In solutions of retinylidenemethylamine (R = CH_3) and retinylideneamines in general, equilibrium (1) favours production of retinene at the concentrations used for spectrophotometry. By greatly increasing the concentration of methylamine it is possible to push the equilibrium in favour of Schiff's base formation, i.e. the method used by Ball *et al.* (1949) in their original preparation of retinylidenemethylamine. Ball *et al.* (1949) used an aqueous solution containing ethanol, but this was found to be unnecessary; in 0·2 M aqueous methylamine at pH 8·6, retinylidenemethylamine does not hydrolyse to retinene.

This will account for the stability of alkaline indicator yellow produced from rhodopsin solution as there will be a large number of amino groups in opsin and other retinal proteins which will prevent hydrolysis of the Schiff's base. Confirmation of this can be found in the report by Collins (1953), that when all free amino groups in rhodopsin preparations were 'blocked' by formaldehyde, alkaline indicator yellow solutions hydrolysed very rapidly. There is therefore no essential difference in the stability of retinylidenemethylamine and of indicator yellow obtained from the eye.

Ball *et al.* (1949) found that methylamine in acid solution will not react with retinene to give retinylidenemethylamine. The most likely explanation of this is that the methylammonium ion is ineffective in equilibrium (1). Methylamine is half ionized at about pH 10·5 (Clark, 1928), and, if this explanation is true, the stabilizing action of a large excess of methylamine on the Schiff's base in alkaline solution should become increasingly less effective as the pH falls. This was tested by making an aqueous solution of retinylidenemethylamine in 0·2 M methylamine and adjusting the pH to values of 6·3 and 3·7. Results are shown in Table 2. Comparing the rates of hydrolysis in the presence and absence of excess methylamine, it is seen that at pH 8·6, excess methylamine prevents hydrolysis; at pH 6·3 it slows hydrolysis, which nevertheless proceeds quite rapidly; at pH 3·7, excess methylamine has

no effect. These findings agree with the suggestion that only un-ionized methylamine is effective in equilibrium (1).

Further, they will explain why, in the absence of added methylamine, the hydrolysis of retinylidenemethylamine slows as the pH rises from 6·6 to 8·6, whereas changing the pH from 8·6 to 13 has a much smaller effect (see Table 2). On putting retinylidenemethylamine into water at pH 13, it breaks down to retinene and methylamine, but the rate of the reaction will be retarded as free methylamine accumulates. In less alkaline solutions, the methylamine formed by the hydrolysis will be converted partly (the extent depending on pH) into methylammonium ions, which will not be effective in slowing the overall rate of breakdown.

If this is true, then any conditions which remove free methylamine from the system will similarly quicken hydrolysis. The breakdown of retinylidenemethylamine was investigated in an aqueous solution of pH 8·6 containing 4% (w/v) formaldehyde, as a trapping agent for amino groups. This produced a very rapid hydrolysis—faster than that recorded at any pH value (see Table 2).

These results all agree with the explanation proposed for the variation in the rate of hydrolysis of retinylidenemethylamine in solutions of different alkalinity. They also clarify the report of Collins (1953) that alkaline indicator yellow hydrolysed much faster than did retinylidenemethylamine. His alkaline indicator yellow was produced in the presence of 30% (w/v) formaldehyde and would therefore appear to break down faster than retinylidenemethylamine, which he studied in a solution containing no formaldehyde.

Although retinylidenemethylammonium salts do not hydrolyse at pH 1, they break down to retinene more easily as the acidity falls. This can be explained by assuming that whereas the uncharged Schiff's base retinylidenemethylamine is readily hydrolysed, the retinylidenemethylammonium ion is stable. At pH 1 substantially all the retinylidenemethylamine will be converted into retinylidenemethylammonium ions, i.e. equilibrium (2) will be forced to the left by the high concentration of hydrogen ions.

$$C_{19}H_{27}CH{=}N^{+}H.R \rightleftharpoons C_{19}H_{27}CH{=}N.R + H^{+}. \quad (2)$$

As the pH of the solution rises, there will be more retinylidenemethylamine itself present, which will hydrolyse to retinene. More retinylidenemethylammonium ions will change over to retinylidenemethylamine and so give retinene until all the retinylidenemethylammonium ions have disappeared. The limiting factor will be the concentration of un-ionized retinylidenemethylamine. The higher the pH, the more retinylidenemethylamine will be present and the faster the apparent hydrolysis of the retinylidenemethylammonium salt to retinene.

The concentrations of the uncharged Schiff's base must be low, as absorption curves recorded during the hydrolysis of retinylidenemethylammonium acetate at pH 3·7 (Fig. 2) show an isosbestic point indicating that only two absorbing substances are present in detectable quantities, i.e. retinylidenemethylammonium acetate and retinene.

These data on retinylidenemethylamine may be used to explain the stability of indicator yellow in solutions prepared from eyes. In alkaline solution the excess of free amino groups in opsin and any other proteins present maintains equilibrium (1) in favour of alkaline indicator yellow, i.e. alkaline indicator yellow is stable. In fairly strongly acid solutions, the high hydrogen-ion concentration maintains equilibrium (2) in favour of the stable acid indicator yellow. In less acid conditions, any uncharged Schiff's base molecules present will hydrolyse to retinene, as in the pH range over which acid indicator yellow will exist, the amino groups necessary for stabilizing any alkaline indicator yellow molecules will have been ionized and thus made ineffective. Therefore in less acid conditions, acid indicator yellow will appear unstable. This theory enables all observations of previous workers on the stability of indicator yellow to be explained in simple chemical terms. There is no need for the postulate (Lythgoe, 1937) of two forms of acid indicator yellow. There are slight differences in the stability of the analogue and of the naturally occurring material, e.g. indicator yellow is stable at pH 3·3 (Lythgoe, 1937), while retinylidenemethylamine will hydrolyse at this pH. Such differences may be expected; the range of stability of these substances will depend on the pK_a of the Schiff's base, which is likely to vary with the amine concerned, and also on the pK_a values of the amino groups in the rhodopsin solutions.

Acid indicator yellow and rhodopsin

Lythgoe (1937) found that if rhodopsin solutions are brought to pH 4·0 in the dark, indicator yellow is formed; that is, acid and light appear to have the same effect on rhodopsin in the conditions used. Collins & Morton (1950) have shown that, when formed in this way, acid indicator yellow must be a direct derivative of rhodopsin. Lythgoe (1937) found the extinction at 440 mμ. after acid destruction of frog rhodopsin to be 0·90 of that initially observed at 502 mμ. in rhodopsin. Wald & Brown (1953) have found ϵ (500 mμ.) of ox rhodopsin to be 40600, and Pitt *et al.* (1950) have reported ϵ (440 mμ.) of retinylidenemethylammonium salts as 41000. The percentage of the retinene present in

rhodopsin which was recovered in indicator yellow can therefore be estimated from the relationship:

$$\text{Percentage recovery} = \frac{40\,600}{41\,000} \times \frac{E_Y}{E_R} \times 100,$$

where E_R = extinction at 500 mμ. of rhodopsin solution, E_Y = extinction at 440 mμ. after acid destruction of rhodopsin (after correcting for dilution).

By using this, it can be calculated from the data of Lythgoe (1937) that by adding an acid buffer of pH 4, 89% of the retinene present in rhodopsin was converted into acid indicator yellow. However, this figure rests on the following assumptions: (1) $\epsilon_{max.}$ of ox rhodopsin is the same as that of frog rhodopsin. (2) $\epsilon_{max.}$ of retinylidenemethylammonium chloride is the same as that of acid indicator yellow. (3) The retinylidene groups of acid indicator yellow have the same proportion of *cis* and *trans* forms as in the solutions of retinylidenemethylammonium chloride used by Pitt *et al.* (1955) to determine the $\epsilon_{max.}$ value used in the above relationship. (4) No alkaline indicator yellow was present in Lythgoe's rhodopsin solutions.

Assumption (2) is probably justified. $\epsilon_{max.}$ values for the indicator yellow analogue agree quite well with those of acid indicator yellow formed by the reaction of retinene with retinal-protein solutions (Collins & Morton, 1950). There is no reason to believe that assumption (1) will introduce any large error, but it is desirable to repeat Lythgoe's work using solutions of ox rhodopsin, in particular by acidifying to a pH below 4, as, in Lythgoe's work, the acid indicator yellow was, of course, unstable. Further, it is essential to exclude the possibility of error arising from the presence of any alkaline indicator yellow in the rhodopsin solution. On acidification, this would change to acid indicator yellow, not derived directly from rhodopsin, and so give misleadingly high figures. Lythgoe was unable to check this point as his spectrophotometer did not read below 395 mμ.

To avoid the presence of alkaline indicator yellow in the rhodopsin solutions prepared for this experiment, rods from ox eyes, after alum treatment, were washed with a buffer of pH 6·3 (converting any indicator yellow into free retinene), dried with anhydrous sodium sulphate and extracted with light petroleum to remove this retinene. Finally, the rhodopsin was extracted with digitonin in a buffer solution of pH 6·3, as retinylideneamines cannot form at this pH.

To the solution of ox rhodopsin buffered at pH 6·3, acid was added bringing the pH to below 1; $\lambda_{max.}$ moved to 440 mμ., showing formation of indicator yellow. This must have come entirely from rhodopsin, since any retinene which might have been present other than in the rhodopsin chromophore would not have been able to form indicator yellow at pH 6·3. Of the retinene present in rhodopsin, 92% was recovered in acid indicator yellow.

To confirm this, it was desirable to check that no appreciable amount of free retinene was formed by the acid destruction of rhodopsin. This was first attempted by bringing the pH to about 6 (when acid indicator yellow hydrolyses to retinene), and estimating the total amount of free retinene present. The method was not very reliable; digitonin began to precipitate in acid solution, causing turbidity with consequent inaccuracies in measurements of extinction. To minimize this source of error, it was found advisable to determine free retinene by using a wavelength on the long-wave side of 400 mμ. The most suitable method was to measure it as a retinylidenemethylammonium salt.

After acid destruction of rhodopsin, the extinction at 440 mμ. was measured, the mixture made alkaline with KOH containing a great excess of methylamine, to convert both the acid indicator yellow and any free retinene into retinylidenemethylamine. The solution was reacidified and the extinction at 440 mμ. again read to determine the amount of retinylidenemethylammonium salt now present. Had any free retinene been formed in the acid destruction of rhodopsin, the second reading of the extinction at 440 mμ. would have been higher (after correcting for dilution) than the first. No such increase was found; the final amount of retinylidenemethylammonium chloride present was 98% of the indicator yellow formed after acid destruction of retinene, a difference which is probably within experimental error.

When rhodopsin is converted by acid into acid indicator yellow, no free retinene, therefore, is formed; all the retinene of the rhodopsin chromophore must be present in acid indicator yellow. The recovery of retinene in indicator yellow would therefore be expected to be 100%. Part of the difference between this and the experimentally found figure of 92% probably arises from the incorrectness of assumption (3) made above. Pitt *et al.* (1955) determined $\epsilon_{max.}$ of retinylidenemethylammonium chloride by dissolving the crystalline *all-trans* form in dilute acid. There is no certainty as to the configuration of the retinylidene group in acid indicator yellow when formed by acid destruction of rhodopsin. It may well contain a proportion of *cis* isomers, as rhodopsin is formed from a *cis*-retinene (Hubbard & Wald, 1952), which will have a lower $\epsilon_{max.}$ than the *all-trans* form. If this is so, then a 100% recovery (in terms of the above relationship) is impossible. It seems likely that this is the cause of the apparently low recovery; when repeated on a different rhodopsin preparation, the experiment gave the same result.

DISCUSSION

Indicator yellow and rhodopsin

Two opinions have been held of the status of indicator yellow: (*a*) that it is a true derivative of rhodopsin, and (*b*) that it is formed by a casual combination of retinene with amino groups. Both views have found supporters. The question has been discussed by Collins (1953), who has shown that

indicator yellow may be a true derivative of rhodopsin. The point is important: the hypothesis that retinene is joined to opsin by a carbon–nitrogen link (Collins & Morton, 1950; Collins, 1953) rests on the assumption that indicator yellow can be derived directly from rhodopsin.

The present study of the stability of retinylidenemethylamine—and by analogy of indicator yellow—should be considered side by side with the findings of Collins & Morton (1950) on the interaction of retinene and retinal proteins. It is perhaps opportune to reassess the nature and status of indicator yellow obtained from rhodopsin solutions.

In alkaline solution, indicator yellow is stabilized by the excess of free amino groups in opsin and in other retinal proteins present as impurities in most rhodopsin preparations. Any retinene formed by hydrolysis of alkaline indicator yellow will immediately reform a Schiff's base by uniting with one of these amino groups, which may well not be the one to which it was joined in rhodopsin.

However, since the ionized amino groups of retinal proteins formed in acid are ineffective in uniting with retinene, any indicator yellow produced from rhodopsin in acid solution must have its retinylidene group still attached to the nitrogen atom involved in the retinene–opsin linkage (Collins & Morton, 1950).

Indicator yellow is, then, a true derivative of rhodopsin provided that it has not at any time passed through alkaline solution containing an excess of amino groups. If it has, it is probably a mixture of various retinylideneamines. This view should harmonize the differing opinions of indicator yellow expressed in the past.

Most of the published work on indicator yellow has been carried out on solutions of rhodopsin. Recently, Arden (1954) has studied the photochemical destruction (bleaching) of rhodopsin in intact rods from frog eyes and has found no evidence of the existence of indicator yellow as an intermediate in the production of retinene. Yet abundant proof exists that indicator yellow can be formed from rhodopsin solutions in suitable conditions. The conclusion to be drawn from Arden's work must be that the pH of the frog rod is within the range in which indicator yellow is not stable. Arden studied the results of bleaching rhodopsin in rods suspended in solutions of various pH values, but still found no difference in the course of bleaching. Presumably putting rods in buffers had no appreciable effect on the pH of the interior of the rod—a likely assumption considering the relative impermeability of the frog rod sheath (Arden, 1954).

Arden's results show that in the visual cycle in the rods indicator yellow is functionally negligible, but that does not detract from the finding that indicator yellow may be a true derivative of rhodopsin.

Collins (1953) showed this to be so by exposing rhodopsin to light in the presence of formaldehyde to prevent recombination of retinene with other amino groups. He was able to detect less than half the indicator yellow expected; the low recovery was due largely to the rapid hydrolysis of indicator yellow in these conditions.

Our repetition of the work of Lythgoe (1937) in the light of more recent knowledge has shown that rhodopsin can be converted directly and quantitatively into acid indicator yellow; *N*-retinylidene-opsin has been formed with the retinylidene group still attached to the nitrogen atom involved in the retinene–opsin link. Provided that the pH is kept down to 1, the retinylidene-opsin ammonium salt formed is fairly stable. This is the first unequivocal demonstration of a direct derivative of rhodopsin stable in aqueous solution at room temperature, and which still contains the opsin portion of the molecule. It is hoped to exploit this to identify the amino group to which retinene is linked in rhodopsin.

Nomenclature

Wald (1953) has objected to the use of the name 'indicator yellow' and urged its replacement by a more 'chemical' term. A revised nomenclature for retinene derivatives was proposed by Pitt *et al.* (1955) and has been used throughout this paper. We suggest that the general name 'indicator yellow' be retained for the mixture of Schiff's bases formed from bleached rhodopsin which has passed through alkaline solution. When indicator yellow is obtained in conditions in which no migration of retinylidene groups can occur, this true derivative of rhodopsin is better named *N*-retinylideneopsin.

The carbon to nitrogen link in rhodopsin

Acid destruction of rhodopsin converts its retinene residue into a substituted ammonium salt form of retinylideneopsin. Every retinylidene group in the salt is joined to a nitrogen atom, and so every retinene residue in rhodopsin must have been linked to opsin by a carbon–nitrogen bond. This bond must readily give rise to the azomethine link of retinylideneopsin. It is difficult to conceive how the —C=N— grouping could be formed from any other carbon–nitrogen linkage in the conditions in which *N*-retinylideneopsin is produced from rhodopsin. The simplest possibility is that it already exists in rhodopsin.

N-Retinylideneopsin has indicator properties because the nitrogen of the azomethine group can alternate between the tertiary and quaternary states. The spectrum of rhodopsin does not change with pH (Lythgoe, 1937), and Lythgoe & Quilliam (1938) have shown that transient orange—in modern

terms, probably a mixture of lumi- and meta-rhodopsin (Wald, Durell & St George, 1950)—does not show indicator behaviour. If, in all these compounds, the terminal carbon atom of retinene is linked to a nitrogen atom of opsin by a double bond, then to account for their lack of indicator properties, the nitrogen atom must be either fixed in the quaternary state or not involved directly in the effective chromophore of rhodopsin.

SUMMARY

1. The stability of retinylidenemethylamine, an indicator yellow analogue, has been studied in aqueous solutions over a range of pH values.

2. It is concluded that retinylidenemethylammonium ions are stable, but that uncharged retinylidenemethylamine molecules hydrolyse to retinene. This hydrolysis is prevented by the presence of excess methylamine, but not by methylammonium ions.

3. These findings explain the influence of pH on the stability of indicator yellow solutions obtained from eyes.

4. On adding acid to rhodopsin solutions in the dark, stable acid indicator yellow solutions can be formed, with the retinene residue attached to the same nitrogen atom as it is in rhodopsin.

5. Essentially all the retinene residues in the rhodopsin chromophore have been shown to be attached to a nitrogen atom of opsin.

6. The status of the different types of indicator yellow formed from rhodopsin solutions is assessed.

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A Modified Conway Unit for Microdiffusion Analysis

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Absorption by the fixative is a difficulty in the microdiffusion method (Conway, 1950) for the determination of small amounts of bromide. Conway recommends a mixture of solid and liquid paraffin as fixative, but points out that the quality of the paraffin is important and that the glass lids should be only lightly smeared. However, it has been found that a definite amount of bromine (sometimes as much as 0·10 m-mole) was always absorbed by the paraffins available here. A similar difficulty with chloride determinations induced Gordon (1952) to use a chlorinated fixative. Pirt & Chain (1952) avoided such small losses of halogen by using a silicone fixative.

A slight modification of the unit is described which avoids any such difficulty and is also more suitable for analytical investigations where relatively high temperatures are required.

EXPERIMENTAL AND RESULTS

The modified unit has an extra chamber (the closing chamber) which is half-filled with the same solutions as the outer diffusion chamber, with the exception of the fluid to be analysed. (In the case of bromide determinations a saturated potassium dichromate solution in 20% (v/v) H_2SO_4 was used.) A liquid trap is formed by dipping an inverted Petri dish into this chamber and thus closing the Unit immediately after running-in the liberating substance. A similar principle of a liquid trap for closing the Unit was developed by Kinsey & Robison (1946), who used oil in the trap. Their Unit was, however, quite different from Conway's and consisted of only one diffusion chamber.

Figs. 1 and 2 show the modified Unit with the cover. In the Unit illustrated in Fig. 2 the closing chamber was made from acrylic plastic (Plexiglass) and attached to the Units by a plastic fixative. If Conway Units are not available for modification, Units containing all three chambers can easily be constructed of one material and no surface requires

grinding. Glass and porcelain have proved to be suitable, while many plastic materials are not always sufficiently inert. Porcelain units of the modified form (Fig. 1) have been supplied by Rudolph Grave, Stockholm.

To open the Unit it is most convenient to suck out the contents of the closing chamber before detaching the Petri dish. As it is sometimes difficult to grip the dish with the fingers, it was grasped with a rubber suction cup and lifted vertically. A piece of filter paper was then interspaced between the dish and the Unit so that it could be put aside without the risk of dropping any liquid into the inner chamber. The analyses then followed the descriptions by Conway (1950).

This modification excludes the possibility of gas leakage, and, as the liquid in the trap is also that used to liberate the volatile substance in the outer diffusion chamber the risk of absorption is eliminated.

Another advantage with this type of Unit is the ease with which it can be cleaned compared with those in which paraffin, vaseline or silicones were used. Washing with soap and then acid could be omitted. Using it for bromide determinations a short soaking in dichromate–sulphuric acid (20%, v/v, H_2SO_4) followed by rinsing in water was sufficient. Generally it is advisable to soak the unit in the same solution as is used for the liquid trap.

The theoretical treatment given by Conway (1950) is not significantly changed by introducing the closing chamber. Only the mean distance of passage in the gaseous phase between the outer and inner chamber will be a little increased, thus making the gradient of the gas tension somewhat lower. As, however, the time for passage in the air space is only a small fraction of the time necessary for the whole diffusion procedure it can be neglected in practice, and the final distribution of the analysed substance will not be significantly changed.

Experience with this new Unit has given excellent results with roughly the same clearance time as with the original Units.

In Figs. 3 and 4 calibration curves for bromide and ammonia determinations are shown. The bromide ion was oxidized by saturated potassium dichromate to bromine, which diffused into a solution of potassium iodide in the central chamber. The liberated iodine was then titrated by thiosulphate. For unknown reasons the calibration curve does not pass through the origin.

The ammonia was formed from an ammonium salt by saturated potassium carbonate and absorbed in a known amount of hydrochloric acid in the central chamber. The ordinate of Fig. 4 shows the difference in the amount of barium hydroxide required to neutralize the total amount of the

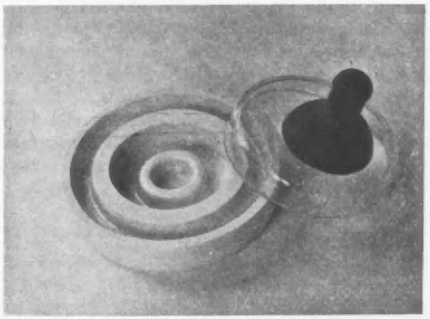

Fig. 1. Modified Unit with Petri dish and rubber suction cup.

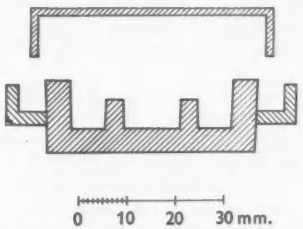

Fig. 2. Cross-section of the Unit and the dish.

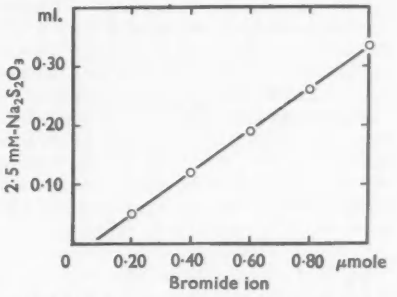

Fig. 3. Calibration curve for bromide determinations.

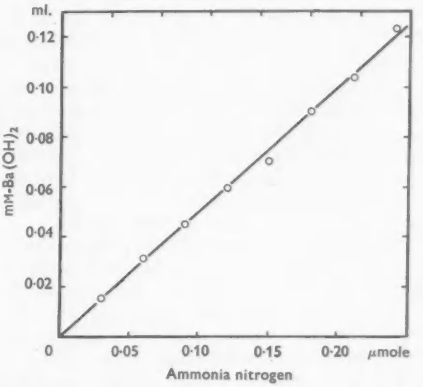

Fig. 4. Calibration curve for ammonia determinations. The ordinate gives the difference in ml. of base required for a blank and sample determination.

hydrochloric acid (blank) and the excess of the acid after absorption of the ammonia.

The proposed modification may be of use in many other analytical procedures involving the use of the Conway microdiffusion method.

SUMMARY

The ordinary Conway Unit has been modified by the addition of a peripheral chamber, to be filled with the same liquid as the outer diffusion chamber (except for the fluid to be analysed). An inverted Petri dish dipped into this chamber forms a liquid trap.

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The Isolation of 16-*epi*Oestriol from the Urine of Pregnant Women

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Many of those who have employed fluorescence reactions with sulphuric or phosphoric acids for the quantitative determination of the oestrogens have noted that phenolic fractions obtained from acid-hydrolysed human urine contain fluorogens other than oestriol (oestra-1:3:5-triene-3:16α:17β-triol), oestrone or oestradiol-17β (cf. Engel, 1950; Zondek & Finkelstein, 1952). While hitherto there has been no evidence to indicate that this 'non-specific' fluorescence is due to substances chemically related to the oestrogens, recent work suggests that it is in part due to substances closely resembling the oestrogens in their solubilities. Thus Migeon (1953), using countercurrent distribution methods, detected three unknown fluorogens in extracts from the urines of normal men and women and patients with hyperactivity of the adrenal cortex, and he showed that these had partition ratios intermediate between those of oestriol and oestradiol-17β in one of the solvent systems employed. More recently Braunsberg, Stern & Swyer (1954) have reported that all samples of crystalline oestriol examined by them contained a fluorogenic impurity which was eluted slightly in front of oestriol itself from partition chromatographic columns in which an alkaline aqueous stationary phase was employed.

Convincing evidence indicating the presence of a substance in human urine, chemically related to, but not identical with oestriol, oestrone or oestradiol-17β, has been obtained using the highly specific Kober reaction (as modified by Brown (1952) and by Bauld (1954)). Our colleague Dr J. B. Brown (personal communication) has detected the presence of a fourth Kober-chromogen in acid-hydrolysed human pregnancy urine by experiments involving chromatography on alumina columns of methylated phenolic fractions. This fourth Kober-chromogen was found by him to be eluted less readily than the 3-methyl ether of oestradiol-17β, but more readily than the 3-methyl ether of oestriol. Working independently, one of us (W.S.B.) detected a fourth Kober-chromogen in the oestriol fraction obtained from the urine of a non-pregnant woman. This unknown chromogen was eluted from partition chromatographic columns somewhat in front of oestriol using the system 70% (v/v) aqueous methanol–ethylene dichloride.

The evidence indicating the presence of a fourth Kober-chromogen (KC-4) in human urine appeared to be so convincing that it was decided to attempt its isolation and identification.

RESULTS

Isolation of KC-4

In the first instance several small batches of pooled pregnancy urine were worked up in order to develop methods for the concentration of KC-4 which could be conveniently used in subsequent large-scale experiments. For the detection of KC-4 in these trial fractionations column partition chromatograms, using the system 70% methanol on Celite–ethylene dichloride (Bauld, 1953), were employed.

Oestriol fractions containing KC-4 were prepared from ether extracts of the acid-hydrolysed urine both by the usual benzene–water partition procedure and by the original procedure of Cohen & Marrian (1934) involving extraction of a 'strong phenolic' fraction by 0·1N-NaOH from ether. The latter procedure, being more economical in solvents, was clearly preferable for large-scale use.

Treatment of such oestriol fractions with cold ethyl acetate removed most of the brown gummy material, leaving a sparingly soluble solid crystalline residue of crude oestriol (Marrian, 1930). This contained KC-4, while little of the latter was found to be present in the ethyl acetate soluble gum. Further concentration of the KC-4 without much loss was achieved by removing part of the oestriol from the crude solid by crystallization from methanol. It may be of interest to note that oestriol recrystallized twice from methanol was free from KC-4 as far as could be determined by the methods employed.

Concentrates prepared in this way still contained, however, about 40 times as much oestriol as KC-4, assuming that the two compounds were equally chromogenic in the Kober reaction; and accordingly it was clear that without further concentration it would not be practicable to use partition chromatogram columns for the large-scale separation of KC-4 from the oestriol, owing to the very low solubility of the latter in nearly all solvents.

In an attempt to achieve further separation of KC-4 from oestriol, countercurrent distribution of the mixture between ether and aqueous alkaline buffer solutions was investigated. It was found that by an eight-transfer operation in separating funnels with a phosphate buffer at pH 12·0 a considerable degree of separation of KC-4 from oestriol could be achieved, the latter behaving in the system as the more 'polar' substance. Furthermore, a good separation of KC-4 from a less 'polar' Kober-chromogen was also achieved. The latter substance was subsequently found to be ketonic, and it seems possible that it was oestrone which had 'leaked' into the oestriol fraction in small amounts. The results obtained by carrying out analytical partition chromatograms on the fractions obtained from the first six 'plates' in the countercurrent separation are shown in Fig. 1.

Although the countercurrent procedure yielded a KC-4-rich fraction from which over 90% of the oestriol originally present had been removed, it still contained too much of the latter to permit of the

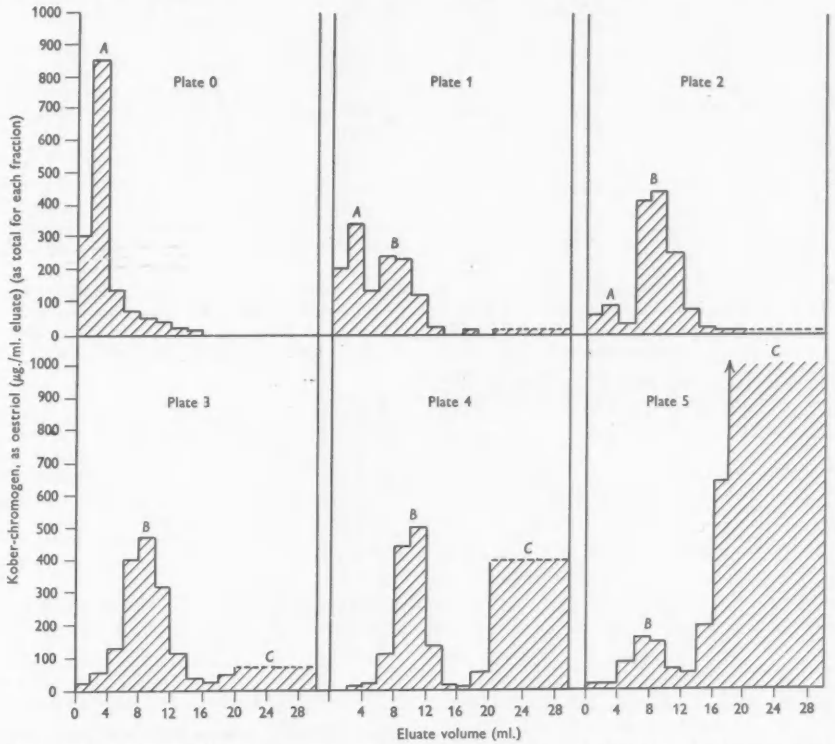

Fig. 1. Analytical column partition chromatograms (70% methanol–ethylene dichloride) on fractions separated from KC-4 concentrate by 8-transfer countercurrent distribution between ether (stationary phase) and pH 12·0 buffer (mobile phase). (Plates 0–5 only are shown.) *A*, 'less polar' Kober-chromogen; *B*, KC-4; *C*, oestriol.

ready preparation of pure KC-4 by direct crystallization. However, owing to the considerable reduction in bulk effected by this procedure it was thought that it might be practicable, even on a large scale, to complete the separation on a partition chromatogram column. This was indeed found to be possible; and when the above-described purification procedures were applied to the 'strong phenolic' fraction from 210 l. of pooled pregnancy urine a KC-4 fraction free from oestriol was obtained (see Fig. 2) which eventually yielded 10·3 mg. of a crystalline product melting at 277–280° and having $[\alpha]_D^{19} + 85°$ (in ethanol).

Identification of KC-4

The product gave C and H analyses in good agreement with those required for a substance of the formula $C_{18}H_{24}O_3$, and on acetylation it yielded a triacetate, m.p. 151–152°. The ultraviolet absorption spectrum in ethanol (ϵ_{281}, 2165) was virtually identical with that of authentic oestriol (ϵ_{281}, 2181; see Fig. 3). The melting points of KC-4 and of its triacetate were markedly depressed by admixture with authentic oestriol and oestriol triacetate respectively. It seemed probable, therefore, that KC-4 was an isomer of oestriol.

On carrying out Kober reactions on KC-4 and authentic oestriol by the procedure of Brown (1952) as modified by Bauld (1954) it was found that while the colours given by the two compounds with a 20 min. heating in the first stage of the reaction were qualitatively almost identical spectroscopically (Fig. 4), that given by KC-4 was somewhat

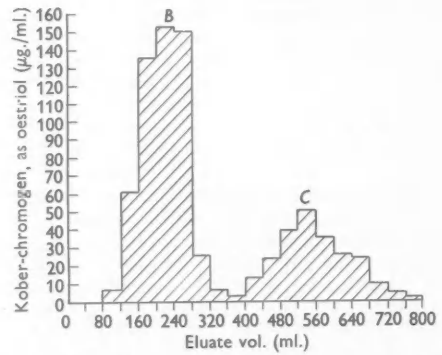

Fig. 2. Preparative column partition chromatogram (70% methanol–ethylene dichloride) on KC-4 enriched oestriol fraction. *B*, KC-4; *C*, oestriol.

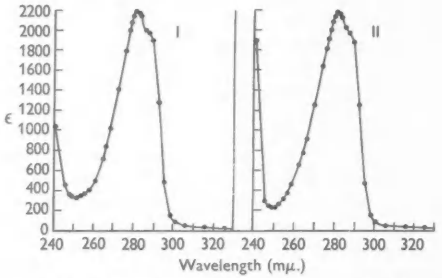

Fig. 3. Ultraviolet absorption spectra of oestriol and KC-4 in ethanol. I. Oestriol: $1{\cdot}66 \times 10^{-4}$ M. II. KC-4: $1{\cdot}74 \times 10^{-4}$ M.

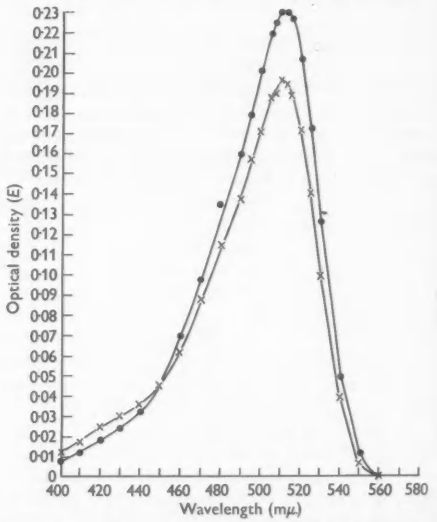

Fig. 4. Absorption spectra of colours developed by oestriol (×) and KC-4 (●) in the Kober reaction with 20 min. heating in first stage. KC-4, 5·01 μg. in final vol. of 3·3 ml.; oestriol, 4·80 μg. in final vol. of 3·3 ml.

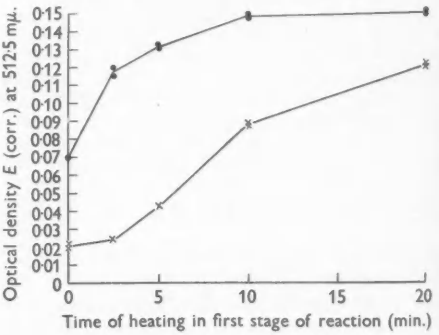

Fig. 5. Effect of variation in time of heating in the first stage of the Kober reaction on intensities of final colours developed by oestriol (×) and KC-4 (●). Concentrations as in Fig. 4.

more intense. Furthermore, when the time of heating in the first stage of the reaction was varied it was found that maximal colour was developed by KC-4 with a much shorter first-stage heating time than was required for oestriol (Fig. 5). In view of the possibility (cf. Bauld, 1953) that the first stage of the Kober reaction with oestriol may involve a dehydration to oestrone, it seemed reasonable to suppose that KC-4 might be a stereoisomer of oestriol which was more sensitive than the latter to the dehydrating action of sulphuric acid—i.e. one of the two stereoisomers with the *cis* configuration of the 16- and 17-hydroxyl groups. In this connexion it appeared significant that 16-*epi*oestriol (Huffman, 1942; Huffman & Darby, 1944; Huffman & Lott, 1947, 1949) was found by Marlow (1950) to give a more intense colour in the Kober reaction than oestriol itself. Subsequently, proof for the presence of a *cis* glycol grouping in KC-4 was obtained by the preparation of an acetonide, m.p. *ca.* 95° and 183–185°.

A comparison of the optical rotation and melting-point data obtained for KC-4 and its derivatives with those reported by Huffman (1942), Huffman & Darby (1944) and Huffman & Lott (1947, 1949) for synthetic 16-*epi*oestriol and its derivatives (see Table 1) suggested the possibility that the two compounds might be identical. An authentic specimen of 16-*epi*oestriol, m.p. 278–281° (our determination), was very kindly supplied to us by Dr Huffman, and on admixture of this with KC-4 the melting point was not depressed. An acetate of the authentic 16-*epi*oestriol was prepared (m.p. 152–154°), and the mixed melting point with KC-4 acetate showed no depression. There can be no doubt, therefore, that KC-4 and 16-*epi*oestriol are identical.

EXPERIMENTAL

Methods

Analytical partition chromatograms. The procedure of Bauld (1953) was followed using 1 × 10 cm. columns with a stationary phase of 5 ml. 70% (v/v) methanol on 5 g. Celite 535, and ethylene dichloride as the mobile phase. The chromatograms were run at a temperature of 18° at a percolation rate of 10 ml./hr. The Celite, methanol and ethylene dichloride were purified by the methods described by Bauld (1953).

In all cases 30 ml. of eluate were collected. In some cases Kober reactions were carried out on the residues obtained by evaporation of successive 2·0 ml. portions of the whole eluate, while in others 2·0 ml. portions were collected only for the first 18 or 20 ml., the remainder being pooled.

Kober reactions were carried out by the method of Brown (1952) as modified by Bauld (1954).

Melting points. All melting points given are uncorrected for emergent stem. Those for oestriol and 16-*epi*oestriol were determined with a conventional type apparatus in sealed evacuated capillary tubes. In this way the decomposition undergone by these compounds when heated in air to just below their melting points was entirely avoided. All other melting points were determined on a microscope hot-stage.

Miscellaneous. The 'pH 10·5 buffer' used for washing ether extracts of the acid-hydrolysed urine was prepared by making up 52 ml. of 5N-NaOH to 400 ml. with 8·5% (w/v) $NaHCO_3$.

The 'pH 12·0 buffer' used for the countercurrent separation was prepared by mixing 250 ml. 0·1M-Na_2HPO_4 with 216 ml. 0·1N-NaOH and making up to 500 ml. with water.

Ultraviolet absorption spectra were determined with a Unicam ultraviolet spectrophotometer SP 500.

All samples for C and H analysis were dried for 4 hr. at 80° *in vacuo* over P_2O_5.

Isolation of oestriol and KC-4 from the urine of pregnant women

Separation of crude crystalline oestriol. Late pregnancy urine (210 l.) was collected without preservative and boiled for 1 hr. with 15 vol. % of 10N-HCl. After saturation with NaCl the mixture was extracted thoroughly with ether, and the extract washed with 'pH 10·5 buffer' to remove acidic substances. Further removal of unwanted impurities was effected by shaking the ether vigorously with a small volume of 2N-NaOH, adding an excess of 8·5% $NaHCO_3$, running off the aqueous layer, and finally washing with water. The washed ether solution was extracted with 0·1N-NaOH, and the 'strong phenolic' fraction recovered from the extract by ether extraction after acidification with H_2SO_4, the ether extract being washed with 'pH 10·5 buffer' and with water before being evaporated to dryness.

To the product (8·38 g.) were added 25 ml. ethyl acetate. After standing overnight at 0°, the crude crystalline material was filtered off, washed with cold ethyl acetate and dried *in vacuo*.

Isolation of authentic oestriol. The crude crystalline oestriol thus obtained (1·36 g.) was dissolved in the minimum volume of boiling methanol (45 ml.) and allowed to stand at room temperature overnight. The crystals deposited were filtered off, washed with cold methanol, and the mother

Table 1. *Comparison of properties of KC-4 and its derivatives with those of* 16-epi*oestriol as reported by Huffman* (1942), *Huffman & Darby* (1944) *and Huffman & Lott* (1947, 1949)

		KC-4	16-*epi*oestriol
Triol	M.p. (°)	277–280	274–276
	$[\alpha]_D$ in ethanol	+85°*	+88°†
Triacetate	M.p. (°)	151–152	152
Acetonide	M.p. (°)	*ca.* 95 and 183–185	103·5–104·5 and 183·5–184·5

* At 19°. † At 29·5°.

liquor and washing were set on one side for the isolation of KC-4. After two further recrystallizations from methanol 120 mg. of oestriol m.p. 273·5–275°, $[\alpha]_D^{19}$ +61° (0·296% in ethanol), ϵ_{281}, 2181, were obtained.

Countercurrent separation of KC-4 fraction. Evaporation of the methanolic mother liquor and washing of the first crystallization of the authentic oestriol yielded 0·975 g. of solid material which was divided into two equal parts for the countercurrent separation. Each part was separately fractionated by an 8-transfer procedure in nine separating funnels using in each funnel 1 l. 'pH 12·0 buffer' and 1 l. ether, the phases having been previously equilibrated with one another. After the completion of the distribution the contents of each funnel were acidified with H_2SO_4, shaken and the aqueous layer was run off. The ether solutions were washed with 'pH 10·5 buffer', 8·5% $NaHCO_3$ and water, and evaporated to dryness. The fractions thus obtained from 'plates' 2–4 (funnels 3–5) were combined for further purification of the KC-4.

Purification of KC-4 fractions by column partition chromatograms. Each of the two KC-4 fractions obtained as described above, after solution in 15 ml. ethylene dichloride, was chromatographed on a column with 80 ml. 70% (v/v) methanol on 80 g. Celite as stationary phase and ethylene dichloride as mobile phase. Twenty successive 40 ml. volumes of eluate were collected, evaporated to dryness, and Kober reactions carried out on appropriate small fractions of each (see Fig. 2). It was evident from the elution patterns that the KC-4 was concentrated in cuts 4–7 from each chromatogram. The material in these cuts from both columns was combined yielding 39 mg. of a nearly white solid.

Isolation of KC-4. The chromatographically purified material was washed with a small volume of benzene at room temperature, and in this way freed from a small amount of colourless gummy material which was discarded. After rapid washing with a small volume of well-chilled methanol, the benzene-insoluble material was crystallized once from aqueous methanol and once from methanol–benzene, yielding 10·3 mg. of white crystals, m.p. 277–280°, $[\alpha]_D^{19}$ +85° in ethanol (*c*, 0·286), ϵ_{281}, 2165. (Found: C, 75·1; H, 8·4. Calc. for $C_{18}H_{24}O_3$: C, 75·0; H, 8·4%.) Mixed with authentic oestriol, the m.p. was 257–270°; with authentic 16-*epi*oestriol, the m.p. was 278–281°.

A further 8·0 mg. KC-4, m.p. 275–277° were recovered from the material in the cold methanol washings and mother liquors of the analytical sample by two crystallizations from methanol–benzene.

Characterization of KC-4

Kober reaction on KC-4 and oestriol: variation in time of heating in first stage of reaction. Solutions of KC-4 and authentic oestriol were prepared by dissolving 0·480 mg. and 0·501 mg. of the two compounds respectively in 10 ml. volumes of ethanol. Into two series of ten test tubes 0·1 ml. of the KC-4 and oestriol solutions respectively were pipetted and evaporated to dryness under an air stream in a boiling-water bath after the addition to each of 50 mg. solid quinol. Pairs of tubes from each series were then treated as follows: after the addition of 2·6 ml. of the oestriol Kober reagent (Brown, 1952; Bauld, 1954) to each tube, the pairs were then heated in a boiling-water bath for x min. (where $x = 0$, 2·5, 5, 10 and 20 for successive pairs from each series). After cooling in cold water for exactly 5 min., 50 mg. solid quinol and 0·7 ml. water were added to each tube and they were then reheated in the boiling-water bath for 15 min. After cooling in cold water for 5 min. the optical densities were measured in a Unicam spectrophotometer SP 600 at 480, 512·5 and 545 mμ., and the corrected density at 512·5 mμ. was obtained by applying the Allen (1950) formula. The curves showing the relationship between optical density at 512·5 mμ. (corrected) and time of heating in the first stage of the reaction are shown in Fig. 5. Absorption spectra over the range 400–560 mμ. for the colours developed by both compounds with a 20 min. heating period in the first stage were also determined (Fig. 4).

KC-4 triacetate. The KC-4 recovered from the mother liquors together with that recovered from the determination of the optical rotation (in all 11·3 mg.) was treated at room temperature for 24 hr. with 0·5 ml. anhydrous pyridine and 0·5 ml. acetic anhydride. After the addition of ice and water the crude acetate was filtered off, washed thoroughly with water and dried. After two crystallizations from hexane, crystals, m.p. 151–152° were obtained. (Found: C, 69·7; H, 7·0. Calc. for $C_{24}H_{30}O_6$: C, 69·5; H, 7·3%.) Mixed with authentic oestriol triacetate (m.p. 126–128°) the m.p. was 107–116°; with authentic 16-*epi*oestriol triacetate (m.p. 152–154°) it was 150–153°.

KC-4 acetonide. To the remainder of the analytical sample of KC-4 (2·2 mg.) was added 1·0 ml. of anhydrous acetone containing 1% (w/v) of HCl (concentration determined by titration after dilution with water). After shaking for a few minutes to dissolve the KC-4, the solution was allowed to stand at room temperature for 30 min. and then diluted with about 10 vol. of ice and water. The insoluble material which separated was filtered off, washed thoroughly with water, dried *in vacuo* over $CaCl_2$ and KOH, and finally crystallized from aqueous methanol. The product melted at about 95° (not sharp), resolidified over the range 124–135° on further heating, and remelted at 183–185° (cf. Huffman & Lott, 1947, 1949).

DISCUSSION

The isolation of 16-*epi*oestriol (oestra-1:3:5-triene-3:16β:17β-triol) from the acid-hydrolysed urine of pregnant women which is described in this paper would appear to be the first recorded isolation of this substance from a natural source. While it is tempting to speculate that the occurrence together of oestriol and 16-*epi*oestriol in urine might point to 16-oxo-oestradiol-17β(3:17β-dihydroxyoestra-1:3:5-trien-16-one) as the common metabolic precursor of both substances, it should be pointed out that the possibility that 16-*epi*oestriol may be an artifact has not been excluded.

It appears probable that the fourth Kober-chromogen detected by one of us in extracts of the urine of a non-pregnant woman was also 16-*epi*oestriol, since its chromatographic behaviour in the system 70% methanol–ethylene dichloride was similar to that of the latter substance in the same system. It is also possible that the fourth Kober-chromogen detected by Brown (personal communication) in methylated phenolic fractions from pregnancy urine was 16-*epi*oestriol 3-methyl ether; but until the behaviour of the latter on alumina

columns has been examined this possibility must remain in doubt. The further possibilities that one of the new urinary fluorogens detected by Migeon (1953) and the fluorogenic impurity in crystalline oestriol detected by Braunsberg *et al.* (1954) may be identical with 16-*epi*oestriol obviously require investigation.

SUMMARY

A new Kober-chromogen has been isolated from acid-hydrolysed human pregnancy urine in a yield of about 0·1 mg./l. It has been shown to be identical with 16-*epi*oestriol (oestra-1:3:5-triene-3:16β:17β-triol).

We gratefully acknowledge a grant from the Medical Research Council from which the cost of this work was defrayed, the generosity of Dr M. N. Huffman of the Oklahoma Medical Research Foundation in sending us a specimen of authentic 16-*epi*oestriol, the skill of Dr J. W. Minnis who carried out the microanalyses, the technical assistance of Miss Winifred Martin and Mr J. Knowles, and the help of Professor R. J. Kellar and Dr T. N. MacGregor of the Department of Obstetrics and Gynaecology, who arranged for the collection of urine.

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The Content and Distribution of Cytochrome *c* in the Fatty Liver of Rats

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Many authors have been interested in the study of the metabolic changes occurring in the liver during experimental fatty degeneration. Though the fundamental metabolic deviation is not yet clear, some phenomena have been described. One of these is the increase of oxygen uptake by liver slices, which was reported by several investigators (Meier & Thoenes, 1933; Califano, 1934; Ennor, 1942; Biagini, 1951). This increase has been recently attributed to a change which takes place in the mitochondria during the fatty degeneration (Dianzani, 1953) and is characterized by an increase of oxidations and by uncoupling of oxidations from phosphorylations. Prader (1947) reported a three-fold increase of the cytochrome *c* content of the rabbit liver which has undergone fatty degeneration as a result of phosphorus poisoning. He used for the determination of cytochrome *c* the method of Prader & Gonella (1947) and attributed the high oxygen uptake of the fatty liver to an increased rate of metabolism. The values for the cytochrome *c* content of normal liver reported by Prader were, however, lower than those obtained by other authors who employed different methods (Rosenthal & Drabkin, 1943; Stotz, 1939; Potter & Dubois, 1942).

The aim of the present study was to determine the content and the distribution of cytochrome *c* in cellular fractions of the liver by two independent methods.

EXPERIMENTAL

Material and methods. Adult albino rats weight 170–180 g. and rabbits weighing 1·5–1·8 kg. were used. They were fed *ad lib.* with a standard diet containing all vitamins and dietary factors. Fatty degeneration of the liver was produced in rats by daily subcutaneous injections of either 0·2 ml. of a 20% (v/v) soln. of CCl_4 in olive oil, or of 0·1 ml. of a 0·5% soln. of white phosphorus in olive oil. Phosphorus was administered for 1–4 days, CCl_4 for 1–60 days. Fatty degeneration in rabbits was produced by injecting 0·5 ml. 0·5% soln. of phosphorus in olive oil for 2–6 days. The animals were killed by bleeding 24 hr. after the last injection; they were kept fasting for 20 hr. before killing in order to reduce (i) the amount of glycogen, which can interfere with the fractionation procedure (Claude, 1946) and the determination of cytochrome *c* (Prader & Gonella, 1947), and (ii) the amount of protein in the mitochondria (Muntwyler, Seifter & Harkness, 1950).

Immediately after the death of the animals, the livers were dissected, weighed, transferred to the cold room at 2°, and 10% homogenates were prepared in a Potter–Elvehjem glass homogenizer with 0·25 M sucrose. Separation of cellular fractions was carried out in the cold room by the procedure

of Schneider (1948). Four fractions ('nuclear fraction', 'mitochondria', 'microsomes', 'supernatant fluid') were collected. The 'nuclear fraction' was washed thrice with sucrose solution and it contained tissue debris, red cells, undamaged cells, nuclei and some mitochondria. The other fractions were only washed once, the washing fluids being added every time to the next fraction. Cytochrome *c* and N-content (Kjeldahl) of each fraction were determined and compared with those of the whole homogenate.

Determination of cytochrome c. Two methods have been used for the determination of cytochrome *c*. The first one was that developed by Prader & Gonella (1947) and the second one was that described by Potter & Dubois (1942).

In the first method cytochrome *c* is extracted by using 1·25 ml. N-H_2SO_4, followed by 0·5 ml. 2N-NH_3/g. tissue. Haemoglobin and some other proteins are removed from the extract by the addition of an equal vol. of sat. $(NH_4)_2SO_4$ and the mixture left at 55° for 20 min. before the precipitate is discarded by filtration. Next, cytochrome *c* is precipitated from the filtrate with trichloroacetic acid (TCA), 1 ml. of 90% (w/v) soln./10 ml. filtrate. After standing in the refrigerator for 16–20 hr. the precipitate is centrifuged, dissolved in water, the solution neutralized with 0·1 N-NaOH and the cytochrome *c* content assessed by determining the difference between light absorption at 550 and 560 mμ. (E_{550}–E_{560}), after reduction with sodium dithionite. The amount of cytochrome *c* was then extrapolated from a curve obtained by the use of a pure preparation of cytochrome *c*, containing 0·46% Fe and kindly supplied by Dr Sven Paléus.

The method of Potter & Dubois (1942) includes extraction of cytochrome *c* with diluted TCA at pH 3·5, neutralization with 1% NaOH, precipitation of cytochrome *c* from the filtrate with concentrated TCA and determination of cytochrome *c* in the dissolved precipitate by means of an enzyme preparation from rat kidney. Light absorption at 550 mμ. is measured before and after addition of 0·01 ml. 0·2M sodium succinate and 0·03 ml. 0·1M-KCN. Calculation is made according to the formula: $c_t = (E_r - E_o)/1{\cdot}91 \times 10^{-7}$, where c_t is the concentration of cytochrome *c* in moles/ml., E_r and E_o the extinctions of cytochrome *c* in the reduced and in the oxidized forms respectively; $1{\cdot}91 \times 10^4$ is the difference between the coefficients of molecular extinction of reduced and of oxidized cytochrome *c*. Spectrophotometric determinations were made with a Beckman Model DU apparatus. The amount of tissue used was 2·5 g. in the case of the method of Prader & Gonella and 1 g. in the case of that of Potter & Dubois. The lipid content of the liver was determined by weighing the dry material, before and after extraction with ether for 4 hr. in a Kumagawa-Suto apparatus. The drying was carried out at 85° to constant weight.

Other methods. Microscopic examination of mitochondria was made with the Zeiss-Winkel phase-contrast microscope. Small pieces of liver were fixed in formalin and used for the preparation of histological specimens, which were stained either with haematoxilin–eosin or with sudan III.

The analytical results were subjected to statistical analysis; standard deviation, standard error of the mean and student's *t* value (Fisher, 1948) were calculated for each group of experiments. The experiments with a '*t*' value corresponding to a probability $P < 0{\cdot}05$ were considered to be significant.

RESULTS

The content and the distribution of cytochrome *c* in the liver fractions of normal rats is shown in Table 1. It is clear from this table that the values obtained with the method of Prader & Gonella are much lower than those obtained with the method of Potter & Dubois. An attempt was made to find the reason for this discrepancy. The method of Prader & Gonella is a spectroscopic one, while that of Potter & Dubois is based on the enzymic reduction of cytochrome *c*. Since, as it was shown by Margoliash (1952, 1954*a*, *b*), the purified preparations of cytochrome *c* contain always a certain amount of enzymically inactive material, the values obtained by the second method would be expected to be lower than those obtained by the first method.

Table 1. *Content of cytochrome c and nitrogen in cellular fractions obtained from the liver of normal rats*

The values, per g. fresh tissue, represent averages (range in brackets) obtained, *A*, by the method of Prader & Gonella (1947) in ten experiments and *B*, by the method of Potter & Dubois (1942) in seven experiments. The values expressed as % denote the percentage distribution of N and cytochrome *c* (Cyt. *c*) in the various fractions as compared with the whole homogenate. The total lipid content of fresh rat liver was 38·8±3·0 mg./g. fresh tissue (range, 35·2–45·3).

			A			*B*		
	Nitrogen		Cyt. *c*			Cyt. *c*		
Fraction	mg./g.	%	μg./g.	%	Cyt. *c*/N	μg./g.	%	Cyt. *c*/N
Whole homogenate	33·0±0·1 (31·2–34·3)	100	28·8±3·8 (23·8–34·3)	100	0·87	113·0±9·4 (99–125)	100	3·4
Nuclear fraction	6·4±0·8 (5·8–7·8)	20·1	6·4±1·1 (5·4–7·9)	26·3	1·0	26·8±4·6 (22–35)	24·9	4·2
Mitochondria	7·3±0·6 (6·2–8·0)	23·0	8·4±2·3 (5·9–11·0)	34·1	1·1	51·9±4·1 (46·0–59·0)	48·3	7·1
Microsomes	5·8±0·6 (5·0–6·9)	18·2	6·7±1·6 (4·5–9·4)	27·4	1·2	16·8±4·0 (10·0–22·0)	15·6	2·9
Supernatant	12·3±1·4 (10·3–15·0)	38·7	3·0±0·8 (2·0–4·0)	12·2	0·24	12·0±2·7 (10·3–18·0)	11·2	0·9
Sum of fractions	31·8	—	24·8	—	—	107·5	—	—
Recovery (%)	96·4	—	85·5	—	—	95·1	—	—

Known amounts of the cytochrome *c* preparation containing 0·46 % Fe were 'determined' by both methods. The values obtained with the procedure of Potter & Dubois were about 50 % lower than those obtained by the method of Prader & Gonella. This means that the percentage of biologically active cytochrome *c* in this preparation was about 50 %. Another preparation containing 0·37 % Fe, prepared according to the method of Keilin & Hartree (1945), contained about 80 % of enzymically active cytochrome *c*.

Since the determinations of cytochrome *c* content in the liver gave higher values with the method of Potter & Dubois than with that of Prader & Gonella, the following experiments were designed to discover at which stage of extraction or purification the losses were occurring. Spectroscopic estimations of the extinction difference between 550 and 560 mμ. in the presence of sodium dithionite were made on the liver homogenate before any treatment and after each stage of both extraction procedures. It was found that both methods extract 95–98 % of the substances absorbing at 550 mμ. In the method of Prader & Gonella a considerable loss, however, occurred during the stage of salting out with $(NH_4)_2SO_4$ at 55°. This agrees with the fact, reported by Paul (1951), that cytochrome *c* becomes less thermostable in the presence of salts. In another type of experiments, a known amount of cytochrome *c* was added to the liver homogenate before extraction. The amount of cytochrome *c* was checked at each stage by reading the difference of extinctions between 550 and 560 mμ. Also in this case a considerable loss occurred as a result of the treatment with $(NH_4)_2SO_4$ at 55° (Table 2).

Differences between the methods of Prader & Gonella and of Potter & Dubois were also observed when determinations of cytochrome *c* were carried out in the cellular fractions. The values for the distribution of cytochrome *c* obtained with the method of Prader & Gonella were 26·3 % in nuclear fraction, 34·1 in mitochondria, 27·4 in microsomes and 12·2 in the supernatant fluid. Those obtained, with the method of Potter & Dubois were 24·9, 48·3, 15·6 and 11·2, respectively. The last results agree with those reported by Beinert (1951), whose figures were 12·8, 56·1, 9·0 and 22·1. Beinert showed that some redistribution of cytochrome *c* takes place in the homogenates during the fractionation. In the present study, the cytochrome *c* of mitochondria never accounted for less than 43 %.

Table 2. *Comparison of two methods for the extraction and determination of cytochrome* c *in rat liver*

The homogenate contained 150 μg. of cytochrome *c*; to this was added 200 μg. of pure cytochrome *c*, and the mixture extracted and purified by *A*, the method of Prader & Gonella (1947), and *B*, the method of Potter & Dubois (1942).

	Cytochrome *c*	
	μg.	% recovery
Method *A*		
Starting material	350	—
Extract	338	96·6
After heating with $(NH_4)_2SO_4$	105	30·0
After precipitation with TCA	100	28·6
Method *B*		
Starting material	350	—
Extract	344	98·3
After NaOH treatment	340	97·4
After precipitation with TCA	320	91·4

Table 3. *Content of cytochrome* c *and nitrogen in cellular fractions obtained from the liver of rats treated with* CCl_4

The values, per g. fresh tissue, represent averages (range in brackets) obtained, *A*, by the method of Prader & Gonella (1947) in seventeen experiments and *B*, by the method of Potter & Dubois (1942) in six experiments. The values expressed as % denote the percentage distribution of N and cytochrome *c* (Cyt. *c*) in the various fractions as compared with the whole homogenate. The total lipid content of fresh rat liver was 62·5 ± 6·6 mg./g. of fresh tissue (range 42·5–82·5).

			A			*B*		
	Nitrogen		Cyt. *c*			Cyt. *c*		
Fraction	mg./g.	%	μg./g.	%	Cyt. *c*/N	μg./g.	%	Cyt. *c*/N
Whole homogenate	29·3 ± 1·8 (27·1–32·9)	100	27·3 ± 3·2 (21·9–32·8)	100	0·93	80·8 ± 5·8 (73·0–90·0)	100	2·72
Nuclear fraction	6·1 ± 0·4 (5·2–7·1)	21·2	6·2 ± 1·3 (3·0–11·9)	24·0	1·0	21·8 ± 3·1 (18·2–27·0)	28·8	3·57
Mitochondria	6·6 ± 0·8 (5·0–8·4)	22·9	7·2 ± 1·9 (3·9–11·9)	27·7	1·1	19·7 ± 3·6 (16·0–24·0)	26·0	2·9
Microsomes	5·1 ± 0·3 (4·0–4·6)	17·7	7·9 ± 1·9 (5·9–10·1)	30·6	1·5	12·6 ± 1·5 (11·0–15·0)	16·6	2·4
Supernatant	11·0 ± 1·2 (9·5–12·9)	38·8	4·6 ± 1·4 (3·2–5·1)	17·7	0·4	21·6 ± 3·9 (18·0–28·0)	28·6	1·96
Sum of fractions	28·8	—	25·9	—	—	75·7	—	—
Recovery (%)	98·2	—	95·0	—	—	93·5	—	—

Table 4. *Content of cytochrome* c *and nitrogen in cellular fractions obtained from the liver of rats treated with phosphorus*

The values, per g. fresh tissue, represent averages (range in brackets) obtained, *A*, by the method of Prader & Gonella (1947) in nine experiments and *B*, by the method of Potter & Dubois (1942) in five experiments. The values expressed as % denote the percentage distribution of N and cytochrome *c* (Cyt. *c*) in the various fractions as compared with the whole homogenate. The total lipid content of fresh rat liver was 61·7±1·8 mg./g. of fresh tissue (range 50·4–76·8).

	Nitrogen		*A* Cyt. *c*			*B* Cyt. *c*		
Fraction	mg./g.	%	μg./g.	%	Cyt. *c*/N	μg./g.	%	Cyt. *c*/N
Whole homogenate	30·1±1·2 (26·9–31·8)	100	23·7±2·2 (13·5–28·3)	100	0·92	76·6±12·3 (59·0–92·0)	100	2·5
Nuclear fraction	6·2±0·7 (5·0–7·0)	20·7	6·1±0·9 (5·4–9·8)	27·2	0·98	19·1±5·6 (12·0–25·0)	23·7	3·1
Mitochondria	6·3±0·4 (5·4–7·0)	21·1	5·0±0·8 (3·1–8·0)	22·3	0·79	21·7±3·3 (16·5–25·0)	27·0	3·44
Microsomes	4·2±0·4 (3·2–4·8)	14·0	6·9±1·5 (4–10·0)	30·8	1·64	11·9±1·6 (10·0–14·0)	14·8	2·8
Supernatant	13·2±0·9 (11·4–14·6)	44·2	4·4±1·7 (2·5–10·0)	19·7	0·33	27·7±2·0 (25·0–29·0)	34·5	2·1
Sum of fractions	29·9	—	22·4	—	—	80·4	—	—
Recovery (%)	99·3	—	94·6	—	—	104·9	—	—

Table 5. *Content of cytochrome* c *in the liver of normal rabbits and of rabbits treated with phosphorus*

The values, expressed in μg./g. (fresh tissue), are averages (range in brackets) obtained, *A*, by the method of Prader & Gonella (1947) in three experiments and *B*, by the method of Potter & Dubois (1942) in three experiments.

	A (μg./g.)	*B* (μg./g.)
Normal rabbits	27·0±4·5 (22·0–31·0)	65·2±6·6 (59·6–72·5)
Rabbits treated with phosphorus	18·7±10·3 (12·5–30·0)	32·6±6·9 (25·5–39·4)

Table 3 shows the distribution of cytochrome *c* in cell fractions from rats treated with CCl_4. The differences between the values obtained with the two methods are quite striking. While the method of Potter & Dubois reveals a significant decrease of cytochrome *c* content of the liver, the values obtained by the Prader & Gonella's method are those encountered in the normal livers. Some difference exists also with respect to the distribution among the cell fractions; with the method of Prader & Gonella the amount of cytochrome *c* found in the supernatant fluid is lower, whereas that found in microsomes is higher than the amounts found in the corresponding fractions analysed by the method of Potter & Dubois. A marked decrease of cytochrome *c* in mitochondria and an increase in the supernatant fluid was found, however, to occur with both methods.

Table 4 shows the results obtained in rats treated with phosphorus. Again in this case a marked difference was observed between the values obtained by the two methods. The main result of the experiments on rats treated with CCl_4 or with phosphorus was that their cytochrome *c* content was not higher than that of normal animals. Since the difference between our results and those of Prader (1947) might have been due to a species difference, some experiments have been carried out on rabbits. Table 5 shows, however, that also in this animal species the cytochrome *c* content of the fatty liver is diminished rather than increased. However, also in this case the values obtained by the method of Prader & Gonella were lower than those obtained by the method of Potter & Dubois.

Experiments were carried out to investigate the cause for the decrease of the cytochrome *c* content in the mitochondria and the increase in the 'supernatant fraction'. Mitochondria from a fatty liver are known to be more frail than those present in the normal liver (Dianzani & Bahr, 1954), and they are known to release into the surrounding medium more ribonucleic acid on 30 min. incubation at 38° than normal mitochondria (Dianzani, 1953). The change in the distribution of cytochrome *c* in the fatty liver may thus be a direct consequence of an increased release of cytochrome *c* from mitochondria. Experiments were made to examine this possibility.

Mitochondria isolated from 2·5 g. of normal and of fatty liver were incubated at 38° for 1 hr. with 10 ml. 0·25 M sucrose. They were then centrifuged 30 min. at 12000 *g* and the cytochrome *c* content of both the sediment and the supernatant fraction was determined. Table 6 shows that the amount of

Table 6. *Release of cytochrome c from normal and from fatty liver mitochondria after incubation at 38° for 1 hr.*

Mitochondria were isolated from 5 g. liver and suspended in 20 ml. 0·25 M sucrose. The suspension was divided into two equal parts. One was immediately analysed for cytochrome *c* by the method of Potter & Dubois, the other after incubation at 38° for 1 hr. The values for normal rats, expressed as μg./g. (fresh tissue), are averages obtained in three experiments. The values for rats treated with phosphorus were obtained in single experiments.

		Cytochrome *c*			
			Incubated sample		
No. of injections of phosphorus	Nitrogen (mg./g.)	Non-incubated sample (μg./g.)	Sediment (μg./g.)	Supernatant (μg./g.)	Released into the supernatant (%)
None	7·2±0·1	64±6	50±2·5	12±2·1	19·3
1	7·4	60	40	18	31·0
2	7·6	56	35·1	19	35·1
4	7·6	40	17	15·4	47·5

cytochrome *c* released from mitochondria isolated from fatty livers is higher than that released from normal mitochondria.

DISCUSSION

The results described in this paper do not favour the conclusion reached by Prader (1947) that the cytochrome *c* content of the fatty liver is higher than that of the normal liver. On the contrary, the content of cytochrome *c* was found lower both in whole homogenates and in mitochondria from fatty livers. Moreover, some redistribution of cytochrome *c* takes place during the fractionation of liver homogenates, and it seems that this redistribution occurs at a higher rate in the fatty liver than in the normal one. Incubation at 38° produces a greater loss of cytochrome *c* from mitochondria isolated from the fatty liver than those isolated from a normal liver. This phenomenon is probably related to the morphological changes occurring in mitochondria as a result of fatty degeneration of the liver.

SUMMARY

1. The content of cytochrome *c* is lower in the liver of rats and rabbits treated with either carbon tetrachloride or phosphorus than in normal animals.

2. Mitochondria separated from a liver which has undergone fatty degeneration contain a smaller amount of cytochrome *c* than those obtained from a normal liver.

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The *N*-Terminal Residues of Bovine Plasma Clot

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The discovery of the non-identity of fibrin and plasma clot obtained by clotting whole plasma in the presence of calcium ions (Robbins, 1944; Lorand, 1948; Laki & Lorand, 1948) served to clear up much of the confusion which surrounded the phenomenon of blood clotting. The determination of the *N*-terminal residues of fibrinogen and fibrin (Bailey, Bettelheim, Lorand & Middlebrook, 1951; Lorand & Middlebrook, 1952) suggested that a similar examination of plasma clot might throw light on the difference between it and fibrin.

MATERIALS

Thrombin. Hoffmann-La Roche's thrombin was used.

Fibrinogen. Citrated bovine plasma was fractionated by precipitation with 0·25-saturated $(NH_4)_2SO_4$ according to Lorand & Middlebrook (1952, section I), except that 0·38% (w/v) sodium citrate was used instead of 0·2% (w/v) sodium oxalate.

Fibrin. This was prepared from the fibrinogen by clotting with a minute amount of thrombin and purifying by precipitation from 30% (w/v) urea solution (Lorand & Middlebrook, 1952).

Plasma clot. Citrated bovine plasma was diluted with 4 vol. of water, then 1 vol. of 0·25M-$CaCl_2$ was added with stirring. The solution was allowed to stand and after approx. 5 min. clotting started. This was allowed to proceed for a further 45 min., then the clot was broken up into small pieces which rapidly contracted and settled to the bottom of the beaker. This precipitate was thoroughly washed for 4 days at 5° with several lots of 0·9% (w/v) NaCl until the washing liquor was free from protein, as judged by precipitation with trichloroacetic acid solution. The plasma clot was then washed with distilled water at 5° until free from chloride ions for two days.

A further sample of plasma clot was swollen in 30% (w/v) urea solution after the NaCl treatment and the solution, maintained at 5°, was replaced 5 times over a period of 3 days. The urea was removed by further washing with distilled water. Both samples of plasma clot were washed in redistilled acetone to remove any fatty material, and finally dried over P_2O_5.

Buffer. The pH 5·0 buffer contained 1·42 g. Na_2HPO_4 and 13·80 g. NaH_2PO_4, H_2O in 100 ml. solution. All reagents were of analytical grade. Eastman's 1-fluoro-2:4-dinitrobenzene (FDNB) was used.

METHODS

Treatment with FDNB. The proteins were treated with FDNB under conditions recommended by Sanger (1945) for 24 hr. except that one-fifth the amount of $NaHCO_3$ was used. The treated proteins were washed with water and ethanol then immersed for approx. 2 hr. in a solution of the disodium salt of ethyienediaminetetra-acetic acid. Finally they were washed in glass-distilled water for 5 hr. and dried.

The diffusate from the plasma clot was treated similarly, but hydrolysed without washing.

Hydrolysis and separation of dinitrophenylamino acids (DNP-amino acids). About 500 mg. of the treated proteins were hydrolysed under reflux in 50 ml. constant-boiling HCl (prepared by distillation). The same flask and boiling-stick were always used, in conjunction with one electrical hot-plate set at the 'low' position. These precautions, together with the treatment with disodium ethylenediaminetetra-acetate and washing with glass-distilled water were believed to contribute to greater reproducibility of the results and less destruction of the DNP-amino acids. The times of hydrolysis ranged from 4 to 24 hr.

For chromatography, a different batch of silica gel was used from that used for the determination of the *N*-terminal residues of fibrin (Lorand & Middlebrook, 1952), and the author found that the properties of the batches differed considerably, even when using buffered columns. On the new gel, DNP-amino acids moved extremely slowly on a pH 6·0 column using ether as the mobile solvent. Therefore the following method of separation was used.

The hydrolysate was extracted 5 times with equal volumes of peroxide-free ether and the pooled ether solutions were evaporated to dryness in a flask. The residue was dissolved in a small amount of $CHCl_3$ and run on a 4 g. column containing 4 ml. pH 5·0 buffer, with $CHCl_3$. The fast-moving decomposition products, including 2:4-dinitroaniline and 2:4-dinitrophenol, were removed but all the DNP-amino acids remained at the top of the column. The flask was rinsed with a small amount of ether, which was transferred to the column, and the mobile solvent was changed to ether. A strong band ($R=1·0$) was obtained, together with extremely weak bands, which were subsequently found to be due to DNP-aspartic acid, DNP-glutamic acid and DNP-serine. The strong band was run on a 2 g. pH 5·0 column with 1% (v/v) ethanol in chloroform. Three bands were obtained: $R=0·26$, found to be *ON*-bis-DNP-tyrosine; $R=0·09$, DNP-glycine; and a weak band which remained at the top of the column, was due to *N*-DNP-tyrosine (a decomposition product) and was removed by 3% (v/v) ethanol in chloroform.

The material in the acid phase was estimated by drying down a portion of the ether-extracted hydrolysate, then

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taking it up in 66% (v/v) methyl ethyl ketone in ether and passing it through a silica-gel column with water as the stationary phase. Only one band could be observed on this column, corresponding to N^{ϵ}-DNP-lysine.

Estimation of DNP-amino acids. The separated DNP-amino acids were made up to 25 ml. or 50 ml. in the appropriate solvent and estimated by means of a Beckman ultraviolet spectrophotometer (Lorand & Middlebrook, 1952). All calculations were based on the dry weight of original untreated protein.

Rates of decomposition of DNP-amino acids. The method used for determining the rates of decomposition of the DNP-amino acids (Table 1) was exactly the same as that used by Lorand & Middlebrook (1952).

In contrast to the previous statement (Lorand & Middlebrook, 1952) the yields of DNP-glycine, corrected for loss by decomposition, were not found to be lower after 6 hr. or 4 hr. hydrolysis. However, the yield of bis-DNP-tyrosine was found to be lower, after 4 hr. (1·4 moles/450 000 g. protein; cf. Table 2).

Protein content of DNP-proteins. The weight of the treated protein after washing and drying was compared with the dry weight of the original protein. The ratio of these two weights was approximately 1·15:1 in all cases. When the treated samples were washed with water for six days the ratio was 1·10:1.

Table 1. *Hydrolytic breakdown of DNP-amino acids by refluxing in constant-boiling* HCl

Amino acid derivative	Time of hydrolysis (hr.)	Measured* between (hr.)	Amount unchanged (%)
DNP-glycine	4	4–8	69·3
	4	8–12	72·7
	4	12–16	69·7
	8	4–12	50·4
	8	8–16	50·8
	12	4–16	35·2
bis-DNP-tyrosine	4	8–12	97·1
	4	12–16	95·0
	8	8–16	92·1
N^{ϵ}-DNP-lysine	24	24–48	94·5

* Two samples of treated protein were hydrolysed for different times. The DNP-amino acids were separated and the percentage decomposition between these time periods was calculated.

Dialysis. Approx. 2 g. samples of fibrin and plasma clot were dissolved or swollen in solutions of monochloroacetic acid, so that the final concentration of the acid was 1% (w/v). These were dialysed in cellophan tubes, first against 1% (w/v) monochloroacetic acid, then against distilled water for 4 days at 5°. The diffusate from the plasma clot was extracted with ether, then evaporated to dryness *in vacuo*.

RESULTS

Plasma clot and fibrin. These proteins gave essentially the same results, and both contained the same ratio and concentration of *N*-terminal residues of glycine and tyrosine (Table 2). This is of interest, in view of the fact that Morrison (1947) showed that plasma clot could coprecipitate with other plasma proteins. Since both the ratio and concentration of the *N*-terminal residues in plasma clot are identical with those of fibrin, it is probable that the prolonged washing with 0·9% NaCl has removed the bulk of these adsorbed proteins. The small amounts of other DNP-amino acids obtained were presumably due to traces of foreign proteins or peptides.

Both proteins contained the same number of free ϵ-amino groups of lysine (Table 4).

Urea-washed and dialysed plasma clot and fibrin. By treatment with dispersing agents such as urea and dialysis with monochloroacetic acid, one *N*-terminal glycyl residue/molecule (average mol.wt. = 450 000) of plasma clot was removed. Preliminary experiments suggest that fibrin is unchanged on dialysis with monochloroacetic acid (Table 2).

The diffusate. In confirmation of the loss of one *N*-terminal glycyl residue from plasma clot, the diffusate was treated with FDNB, hydrolysed and found to contain a high concentration of DNP-glycine together with traces of other DNP-amino acids.

Prolonged washing of DNP-protein. As it was noticed that the wash liquor was always coloured yellow, in spite of many changes of water, additional samples of DNP-protein were repeatedly washed

Table 2. *Estimated number of moles of DNP-amino acids from* 450 000 *g. of protein, after correction for hydrolytic breakdown (time of hydrolysis (hr.) in brackets)*

	DNP-aspartic acid	DNP-glycine	Bis-DNP-tyrosine
Fibrin	Traces	4·3 (4)	2·0 (8)
Fibrin (dialysed)	Traces	3·7 (4)	—
		3·8 (8)	2·0 (8)
Plasma clot	Traces	3·9 (4)	—
		4·1 (8)	2·3 (8)
		3·7 (8)	2·2 (8)
Plasma clot (dialysed)	—	3·3 (8)	1·9 (8)
Plasma clot (urea-washed)	Traces	3·1 (4)	2·2 (4 + 4)*
	0·003 (8)	3·0 (8)	2·0 (8)
		3·2 (12)	2·0 (12)

* 4 hr. hydrolysis followed by further 4 hr. hydrolysis of peptide material.

Table 3. *Effect of prolonged washing of DNP-protein on the* bis-*DNP-tyrosine content of fibrin and plasma clot*

	Time of washing (hr.)	Moles bis-DNP-tyrosine/ 450000 g. of protein
DNP-plasma clot	0	2·0
	6	2·1
	144	0·8
DNP-fibrin	6	2·0
	144	0·9

Table 4. *Lysine content derived from the* N^{ϵ}*-DNP-lysine estimations after* 24 *hr. hydrolysis*

	Moles lysine/450000 g. of protein
Fibrinogen	195
	196
Fibrin	206
Plasma clot (urea-washed)	195
	198

with several lots of distilled water for 6 days. The final washings were still yellow, suggesting a slow evolution of 2:4-dinitrophenol from the *O*-DNP group of bis-DNP-tyrosine. This was confirmed by determination of the bis-DNP-tyrosine content of the hydrolysate, which was low, with a corresponding increase in the amount of *N*-DNP-tyrosine (Table 3). A sample of FDNB-treated plasma clot was hydrolysed, therefore, without washing to see if 6 hr. washing had any effect on the bis-DNP-tyrosine content. No effect was found, but a small amount of *N*-DNP-tyrosine was obtained even in this case. This was attributed to the slight decomposition of the bis-DNP-tyrosine during acid hydrolysis, which is normally corrected for. The length of time of washing had no effect on the estimation of the *N*-terminal glycyl residues.

DISCUSSION

Nanninga (1946) and Edsall, Foster & Scheinberg (1947) obtained a value of 450000 for the molecular weight of bovine fibrinogen. Recently, Katz, Gutfreund, Shulman & Ferry (1952) and Shulman (1953) calculated this to be 340000. Presumably, these would be maximal values for the molecular weights of fibrin and plasma clot. The present determinations are more in accord with the first value, for both fibrin and plasma clot.

The previous finding (Lorand & Middlebrook, 1952) that the molecule of fibrin of weight 450000 has two *N*-terminal tyrosyl and four *N*-terminal glycyl residues was confirmed. This suggests that the molecule possesses six open peptide chains, not necessarily of equal length. Although plasma clot, presumed to have a similar molecular weight, is precipitated with two *N*-terminal tyrosyl and four *N*-terminal glycyl residues, it seems that the molecule has really two tyrosyl and only three glycyl residues. Thus the molecule possesses five open peptide chains. The sixth chain, possessing an *N*-terminal glycyl residue (a product of the conversion of fibrinogen into fibrin by thrombin) (Lorand & Middlebrook, 1952), is strongly adsorbed on the plasma-clot molecule, but can be removed by 30% (w/v) urea or monochloroacetic acid solution. The molecular weight of this chain is sufficiently small to allow diffusion through a cellophan membrane. It was detected in the diffusate by reaction with FDNB and separation of DNP-glycine after acid hydrolysis.

Within the limits of experimental error, fibrin and plasma clot contain the same number of free ϵ-amino groups of lysine, as revealed by the FDNB method.

SUMMARY

1. Bovine fibrin and plasma clot were treated by the dinitrophenyl method and the *N*-terminal residues determined.

2. Like the fibrin molecule, that of plasma clot has two *N*-terminal tyrosyl residues, but three instead of four *N*-terminal glycyl residues.

3. Plasma clot is precipitated with four chains having *N*-terminal glycyl residues, but one of these can be removed by urea or monochloroacetic acid solution.

4. Fibrin and plasma clot contain the same number of free ϵ-amino groups of lysine.

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The Determination of ^{65}Zn in Tissues with Geiger and Scintillation Counters

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^{65}Zn has been used in several biological investigations (see, for example, Sheline, Chaikoff, Jones & Montgomery, 1943*a*, *b*; Heath, 1949; Andersson-Kottö & Hevesy, 1949; Banks, Tupper & Wormall, 1950; Heath & Liquier-Milward, 1950; Rosenfeld & Tobias, 1951; Tupper, Watts & Wormall, 1951, 1952, 1954), and since radioactive zinc of high specific activity is now more readily available, it is probable that this isotope will be more widely used in biological studies. As far as we are aware, all the previous workers using ^{65}Zn for biological experiments have made their radioactivity measurements with some type of Geiger–Müller counter. A suitable scintillation counter system, employing a thallium-activated sodium iodide crystal as the phosphor, has been developed, and found to possess several advantages over Geiger–Müller counters for this type of work.

The present paper describes simplified methods for determining ^{65}Zn in tissues using the Veall (1948) type Geiger–Müller counter for liquids, and the scintillation counter. A preliminary note on some of this work has been published elsewhere (Banks, Tupper, Watts & Wormall, 1954).

MATERIALS AND METHODS

Radioactive zinc (^{65}Zn). This was obtained either in the form of neutron-bombarded zinc metal or from copper which had been irradiated in a cyclotron. Improved methods for separating ^{65}Zn from the latter source have been described elsewhere (Tupper & Watts, 1954; Tupper *et al.* 1954).

Geiger-Müller counters. These were of the type described by Veall (1948) for measurements on liquid samples (M6, M6H or M6M; 20th Century Electronics Ltd.).

Scintillation counter. The arrangement and housing of the radioactive sample, the phosphor (a thallium-activated NaI crystal, supplied by Atomic Energy Research Establishment, Harwell), and the photomultiplier tube (EMI 6260) are shown in Fig. 1. It is essential to have good optical contact between the base of the phosphor mounting and the photocathode of the electron multiplier, and this is effected with a thin film of liquid paraffin. The voltage pulses set up in the output resistor of the photomultiplier are fed to a two-stage amplifier (giving a 50-fold gain) situated in the base of the instrument. The output pulses from the amplifier are fed to a decade-type scaling and recording unit (Panax Equipment Ltd., 'Model 100 C'). The 'dead time' of this system is only of the order of a few microseconds, and consequently dead time corrections are rarely needed in this work. Aluminium filters (with a total thickness equivalent to 480 mg./cm.^{2}) are incorporated into the crystal holder, and the base of the sliding tray which holds the glass dish containing the radioactive sample. This ensures that only the γ-rays produced by the radioactive decay of ^{65}Zn reach the activated NaI crystal.

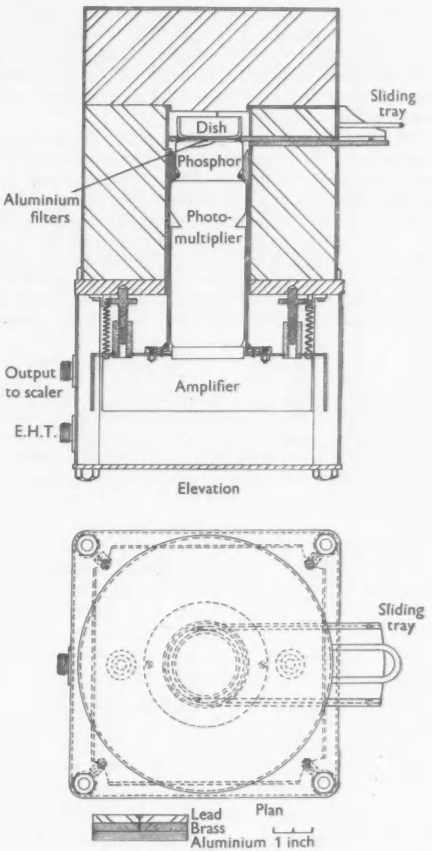

Fig. 1. Scintillation counter.

The high-voltage power supply for the photomultiplier is drawn from an oscillator type 'power-pack' incorporated in the above-mentioned 'Model 100 C' unit.

After preliminary experiments to determine the optimum pulse discrimination, we were able to obtain reproducible counting rate/voltage characteristic curves, with plateaux having a gradient of about 1%/100 v. From considerations of the stability of the background, stability of E.H.T. (extra high tension) supply, and counting rate/voltage characteristic, an operating voltage of 1500 was chosen for routine work.

METHODS

A shortened wet combustion procedure. A weighed portion (0·1–5 g.) of the material to be investigated is heated with conc. H_2SO_4 or HNO_3 (2 ml./g. tissue, with a minimum of 1 ml.) until a clear solution is obtained. Further portions of acid are added if necessary. When H_2SO_4 is used, SeO_2 (about 5 mg./ml.) should be added and the heating continued for 0·5 hr. after the solution turns clear; part of this digest can then be used for a total nitrogen determination. The acid digest is diluted to a standard volume (e.g. 15 or 20 ml.) with water, and a portion of this solution used for radioactivity measurements with either a liquid counter or the scintillation counter. Digests prepared in this way from normal tissues showed no significant radioactivity under these conditions.

Extraction method. It may be necessary, under certain circumstances, to separate the zinc chemically from other tissue components before its radioactivity is measured. The method of Banks *et al.* (1950) is suitable for this purpose.

The use of the scintillation counter. Tissues (or other biological materials, e.g. faeces) are homogenized, or are digested with H_2SO_4 or HNO_3 as described above. A portion (e.g. 5·0 ml.) of the homogenate or acid digest is placed in a circular glass dish (depth 1 cm., diameter 3 cm.), and the dish is moved to a predetermined and reproducible position above the phosphor by means of a sliding metal carrier (see Fig. 1).

Sufficient material to cover the whole of the bottom of the dish of the scintillation counter should always be used, and it is advisable to maintain an approximately constant volume for any one series of measurements.

RESULTS

Recovery of added ^{65}Zn

Measured amounts of ^{65}Zn were added to samples of normal rabbit tissues. These samples were then digested by sulphuric acid containing selenium dioxide, and the radioactivity of the diluted digests was determined in some experiments with the Veall-type liquid counter and in others with the scintillation counter described above. In all these experiments the recovery of added ^{65}Zn was essentially complete (Table 1).

In many of our investigations with ^{65}Zn it has been found necessary or more convenient to make radioactivity measurements on unhydrolysed tissue homogenates, and tests have therefore been made to determine whether, using the scintillation counter, an accurate measure of the ^{65}Zn content of a tissue can be obtained in this way. The results have shown that determinations made in this simple way are completely reliable.

In a few experiments, samples of normal rabbit liver, kidney and muscle, containing added ^{65}Zn, were digested with conc. HNO_3, and the radioactivity of the diluted digests was measured in a Veall-type Geiger–Müller counter. The recovery of added ^{65}Zn in a typical experiment was 97, 97, 98,

Table 1. *Recovery of* ^{65}Zn *added to normal tissues*

For Expts. 1 and 2 a solution containing ^{65}Zn (amounts giving count rates of 2184 and 1226/min. with the counters used in Expts. 1 and 2, respectively) was added to about 5 g. of rabbit tissue. For Expt. 3 normal rabbit liver was mixed with about an equal volume of 0·9% NaCl and the mixture was disintegrated in a Waring Blendor and a solution containing ^{65}Zn (giving a count rate of 22176/min. with the appropriate scintillation counter) was added to each 5 ml. sample. All the samples were digested with H_2SO_4 containing SeO_2.

	Counter	Tissue	Density of the diluted acid digest (g./ml.)	^{65}Zn recovered (%)
Expt. 1	Geiger–Müller counter for liquids	Liver	1·15	100
			1·19	99
		Kidney	1·23	99·5
			1·23	99
		Muscle	1·24	101
			1·22	96
Expt. 2	Scintillation counter	Liver	1·22	100
			1·23	97
		Kidney	1·19	101
			1·23	100
		Muscle	1·15	103
			1·24	101
Expt. 3	Scintillation counter	Liver	—	98
			—	98
			—	100

98, 98 and 101 %, with the diluted digests having, respectively, densities of 1·26, 1·30, 1·13, 1·34, 1·26 and 1·20 g./ml.

The linearity of the response of the scintillation counter was investigated by comparing the known activity of a series of ^{65}Zn solutions with the count rates obtained using the scintillation counter. The known radioactivity of the solution and the observed count rates were directly proportional to one another at count rates not exceeding 30 000 counts/min. Above this count rate the observed value was slightly less than the calculated value, and this small deviation from a linear relationship at higher count rates is probably due to the inertia of the mechanical register in the scaler employed.

Self-absorption effects

As we wished to make direct radioactivity determinations on the acid digests containing varying amounts of solid matter, and often having relatively high densities, a series of experiments was undertaken to determine whether 'self-absorption' effects were likely to vitiate the results obtained with the liquid counter. Various ^{65}Zn-containing solutions having densities as high as 1·83 g./ml. were studied, including solutions of sulphuric and nitric acids, zinc sulphate, and sodium iodide, and a concentrated casein solution digested with trypsin, and in no case was there any appreciable 'self-absorption'. These results are in agreement with those obtained in recovery experiments (cf. Table 1). Similar results were obtained with the scintillation counter.

Direct comparison of the results obtained by the shortened wet combustion procedure and those obtained by the method of Banks et al. (1950)

Having established that the shortened wet combustion method gives complete recovery of ^{65}Zn added to normal tissues, we made further tests with tissues into which ^{65}Zn had been biologically incorporated, to compare the results with those obtained by the longer method (Banks *et al.* 1950) which involves complete digestion of the tissue by long heating with various acids and subsequent extraction of the zinc by dithizone (diphenylthiocarbazone).

The results obtained with various tissues (liver, kidney and lung) from a rabbit which had received an intravenous injection of ^{65}Zn (as a zinc–glycine complex) 5 days previously, showed that the simplified method gives reasonably reliable results with ^{65}Zn-containing tissues, even when, as in this particular experiment, the amount of ^{65}Zn present is small.

Comparative count rates for ^{65}Zn *using (a) the Veall-type Geiger–Müller counter and (b) the scintillation counter*

The radioactivity of a number of ^{65}Zn-containing solutions was measured with the Geiger–Müller counter for liquids and with the scintillation counter, the samples used for the scintillation counter measurements being suitably diluted. The results of these determinations are given in Table 2.

Table 2. *Comparative count rates for* ^{65}Zn *sources measured with a Geiger–Müller liquid counter and with the scintillation counter*

For experimental details see this page.

Sample	Radioactivity (net counts/min./10 ml. solution) Geiger–Müller counter (*A*)	Scintillation counter (*B*)	Ratio (*B*/*A*)
1	814	19 176	23·8
2	1 604	37 136	23·1
3	3 269	75 300	23·0
4	6 600	158 834	24·0
5	8 130	193 240	23·8
6	11 040	250 300	22·8

DISCUSSION

The method for determining the ^{65}Zn content of tissues which we have hitherto used (Banks *et al.* 1950) is laborious and it involves considerable manipulation and transference of solutions, with a loss of 3–5 % of the ^{65}Zn. The present paper describes much simpler methods which can satisfactorily be used for most biological investigations with ^{65}Zn. Where the tissue samples contain significant amounts of other radioactive isotopes, however, the longer procedure of Banks *et al.*, involving complete digestion of the tissue by acid and subsequent extraction of the zinc, should be followed.

Our first aim was to modify the original method by omitting the time-consuming extraction of the zinc, but since acid digests of tissues contain varying amounts of solid matter it was first necessary to establish that there was no appreciable self-absorption when the radioactivity of these ^{65}Zn-containing acid digests was measured in a Veall-type liquid counter. Veall (1948) has shown that significant self-absorption occurs, with solutions having densities as low as 1·03 g./ml., when this counter is used for measuring the radioactivity of ^{40}K and ^{32}P, but our investigations have shown that there is no similar effect with ^{65}Zn, even when the density of the solution was considerably greater than that of the most concentrated tissue digest. It was, therefore, considered justifiable to use simple acid digests of tissues for ^{65}Zn determinations, and the results presented in Table 1 confirmed this view.

The absence of any detectable self-absorption effect in these experiments with the Veall-type liquid counters indicates that these counters were functioning as γ-ray counters, the soft positrons (β^+) and K-capture X-rays emitted during the radioactive decay of ^{65}Zn being completely absorbed by the solution itself and the glass wall of the counter tube (25–30 mg./cm.^{2}).

For the preparation of the acid digest of a tissue, either sulphuric acid (plus selenium dioxide) or nitric acid may be used, but the former has several advantages over the latter; the sulphuric acid digest can be used also for a total nitrogen determination, and this is particularly helpful in many biological investigations with ^{65}Zn. Digestion of the tissue with nitric acid to give a homogeneous solution is appreciably quicker than digestion with sulphuric acid and may be preferred when large tissue samples are being analysed, but with nitric acid there is a much greater risk of corrosion of the radioactivity measuring apparatus by acid fumes. Furthermore, material is frequently precipitated when the nitric acid digests of tissues are diluted.

A scintillation counter has a high efficiency for the detection of γ-rays and would therefore be expected to be a more sensitive instrument than the Geiger–Müller counters used for the detection and measurement of ^{65}Zn, 45 % of the disintegrations of which results in γ-ray emission (Hollander, Perlman & Seaborg, 1953). The prediction was confirmed by the results of the many parallel observations, typical examples of which are recorded in Table 2, the scintillation counter being about 23 times as sensitive as the Veall-type Geiger–Müller counter.

The accuracy which can be attained in a radioactivity measurement depends on the ratio of the (net count rate of the radioactive source)/(background count rate). Although the background count rate obtained with the scintillation counter is considerably larger than that obtained with the Geiger–Müller tubes used, these being about 150 and 12 counts/min. respectively, the (net count rate of sample)/(background count rate) ratio is larger, and therefore more favourable, than the corresponding ratio for the Geiger–Müller counters. Also, since larger counting rates are given with the scintillation counter, it is often possible to obtain a desired statistical accuracy in a shorter time.

A further advantage of the scintillation counter method is that the glass dishes in which the final solution is placed for radioactivity measurements are of simple design and are easily cleaned. They are less likely to become seriously contaminated than are the Geiger–Müller counters for liquids, and since a separate dish can be used for each sample, many radioactivity measurements can be made in a short time. The method is of general application and has given satisfactory results with unhydrolysed samples of ^{65}Zn-containing egg yolk, blood and homogenized faeces.

SUMMARY

1. A simplified method of determining the ^{65}Zn content of tissues, using the Veall-type liquid counter, is described.

2. A scintillation counter suitable for the measurement of ^{65}Zn present in fluids or tissue homogenates is described.

3. The special advantages of the scintillation counter in biological work with radioactive isotopes are briefly discussed.

The expenses of these investigations were in part defrayed by grants from the British Empire Cancer Campaign and the Medical Research Council. The thallium-activated sodium iodide crystal was obtained from the Atomic Energy Research Establishment, Harwell, and we appreciate the help which we received from Dr D. Taylor and Mr R. B. Owen in this matter. We are also greatly indebted to Professor P. B. Moon, F.R.S., and Professor W. E. Burcham for the arrangements under which we were able to obtain irradiated copper targets from the cyclotron at Birmingham University, and to Mr L. W. Blow for constructing the scintillation counter.

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Studies in Detoxication

60. THE METABOLISM OF ALKYLBENZENES. *ISO*PROPYLBENZENE (CUMENE) AND DERIVATIVES OF HYDRATROPIC ACID

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*iso*Propylbenzene (cumene) is used as a solvent for cellulose lacquer, although not extensively. It has narcotic properties but no toxic effects in man have been reported (see Browning, 1953). When fed to rabbits it causes the excretion of considerable amounts of conjugated glucuronic acid and the urine reduces Benedict's reagent (Smith, Smithies & Williams, 1954*a*), but nothing definite is known concerning the nature of the metabolites of cumene. The earlier work of Nencki & Giacosa (1880) and of Thierfelder & Klenk (1924) showed that it did not give rise to hippuric acid or any other aromatic acid. However, the former authors found that the ratio of inorganic sulphate to ethereal sulphate in the urine of the dog was reduced after administering cumene, and on these grounds suggested that a phenol was formed (cf. Donahue & Harris, 1945), but Smith *et al.* (1954*a*) found that in the rabbit ethereal sulphate formation was very small.

Our work on ethylbenzene (Smith, Smithies & Williams, 1954*b*) has suggested that in this compound biological oxidation occurs initially mainly at the active methylene group next to the benzene ring. We were, therefore, particularly interested in whether a similar oxidation would occur with cumene giving rise to 2-phenylpropan-2-ol ($Ph.C(OH)(CH_3)_2$). We shall show that this is, in fact, true and that cumene gives rise also to hydratropic acid (α-phenylpropionic acid) and hydratropyl alcohol (2-phenylpropan-1-ol).

EXPERIMENTAL

Materials. The following compounds were purchased: cumene, b.p. 152–153°; (±)-hydratropic aldehyde, b.p. 205°; α-methylstyrene, b.p. 165°. 2-Phenylpropan-2-ol (dimethylphenylcarbinol), b.p. 202°, was prepared from bromobenzene and acetone according to Tissier & Grignard (1911). (±)-Hydratropic alcohol, b.p. 113–114°, was prepared in excellent yield by the reduction of hydratropic aldehyde with lithium aluminium hydride by standard methods (cf. Brown, 1951). (±)-Hydratropic acid, b.p. 265°, was prepared according to Arcus & Kenyon (1939) from the aldehyde.

Animals. Chinchilla rabbits kept on a constant diet were used. All compounds were administered suspended in water by stomach tube.

Table 1. *Glucuronic acid conjugation of hydratropic acid and related compounds by the rabbit*

Figures in parentheses are average values.

Compound fed	Dose (g./kg.)	Conjugation (% of dose)
(±)-Hydratropic alcohol	0·5	60, 78, 80 (73) 68*, 66* (67*)
(±)-Hydratropic aldehyde	0·3	51*, 52*, 54* (52*)
(±)-Hydratropic acid	0·3	63*, 72*, 100* (78*)
(+)-Hydratropic acid†	0·3	73*, 74*, 90* (79*)
(−)-Hydratropic acid‡	0·3	58*, 66*, 67* (64*)
2-Phenylpropan-2-ol	0·5	60, 97, 98 (85)
Cumene	0·45	68§

* Ester glucuronide as shown by titration with Benedict's reagent.
† 95% (+)-isomer.
‡ 77% (−)-isomer.
§ Figure quoted from Smith *et al.* (1954*a*).

Methods. Total glucuronic acid in urine was determined by the modified naphthoresorcinol method described by Paul (1951). Ester glucuronide was estimated by titrating in the usual way with Benedict's reagent standardized against hydratropoylglucuronide (see Table 1 for results).

Experiments with hydratropic acid and its derivatives

Hydratropoylglucosiduronic acid and its derivatives

From (±)-hydratropic aldehyde. A total of 18 g. of the aldehyde was fed to six rabbits, and the urine, which reduced Benedict's and Fehling's solutions readily and gave a strong naphthoresorcinol test, was collected for 24 hr. The glucuronide gum was prepared through the basic lead salt (cf. Kamil, Smith & Williams, 1951). The gum was dissolved in a little methanol, and 250 ml. ether followed by 100 ml. of light petroleum (b.p. 40–60°) were added. This caused the separation of a gummy precipitate *A* which yielded no crystalline material on methylation and acetylation. The filtrate from *A* was treated with a further 100 ml. of light petroleum and the gum *B* which separated was methylated with ethereal diazomethane. On evaporation of the solvents and recrystallization of the residue from water *methyl (+)-hydratropoyl-β-D-glucosiduronate dihydrate* (*methyl (+)-α′-phenylpropionyl-β-D-glucosiduronate*), 0·7 g., separated as colourless needles, m.p. 165–166° and $[\alpha]_D^{20}+22°$ in $CHCl_3$ (*c*, 1). (Found: C, 51·3; H, 6·3; OMe, 8·3. $C_{16}H_{20}O_8$, $2H_2O$ requires C, 51·1; H, 6·4; OMe, 8·4%.) It reduced Fehling's and Benedict's reagents on warming. By adding

more light petroleum to the filtrate from gum *B*, more gum separated and this on methylation yielded a further 0·8 g. of the methyl ester (total yield, 1·5 g. or about 6% of dose). Acetylation of this ester with pyridine and acetic anhydride and then pouring the mixture into water yielded *methyl (+)-hydratropoyl-tri-O-acetyl-β-D-glucosiduronate* which formed needles from ethanol, m.p. 130–132° and $[\alpha]_D^{20} - 10°$ in $CHCl_3$ (*c*, 1). (Found: C, 56·7; H, 5·6. $C_{22}H_{26}O_{11}$ requires C, 56·6; H, 5·6%.) This compound also reduced Fehling's and Benedict's reagents on heating.

The above experiment was repeated with 6 g. of hydratropic aldehyde to the stage of removal of the gummy precipitate *A*. The filtrate from this was then treated with more light petroleum and the whole allowed to stand several days at 0°. A crystalline solid (50 mg.) separated and was crystallized from water. This was *(+)-hydratropoyl-glucosiduronic acid* (*(+)-α′-phenylpropionyl-β-D-glucosiduronic acid*), which formed colourless needles, m.p. 163–164° and $[\alpha]_D^{20} + 23°$ in ethanol (*c*, 1). (Found: C, 53·3, 53·2; H, 6·2, 6·1. $C_{15}H_{18}O_8$, 0·5 H_2O requires C, 53·7; H, 5·7%.) The glucuronide readily reduced Benedict's reagent, and on methylation and acetylation yielded the same triacetyl methyl ester (m.p. 132° and $[\alpha]_D^{20} - 10°$ in $CHCl_3$) as that described in the preceding paragraph.

The positive rotation of the hydratropoylglucosiduronic acid and its methyl ester suggested that we were dealing with the glucuronide of (+)-hydratropic acid, although the (+) and (−) forms could be expected from (±)-hydratropic aldehyde. Hydrolysis of the methyl ester (1 g.) with 2N-H_2SO_4 yielded, after ether extraction, evaporation and distillation, 250 mg. of an acidic oil (hydratropic acid) with $[\alpha]_D^{20} + 72·3°$ in ethanol (*c*, 1·5). In another experiment (+)-hydratropamide was isolated as follows. Methyl hydratropoylglucosiduronate, m.p. 165–166° (0·9 g.) in 2N-H_2SO_4 (5 ml.) was heated on a water bath for 4 hr. After cooling, the solution was extracted with ether, the extract evaporated and the residue distilled *in vacuo*. The acidic distillate (220 mg.) was converted into the amide (cf. Wild, 1947) which was recrystallized from benzene–*n*-hexane. The (+)-hydratropamide (80 mg.) had m.p. and mixed m.p. 97° and $[\alpha]_D + 52°$ in $CHCl_3$ (*c*, 1) and was identical with a sample of authentic (+)-hydratropamide, m.p. 97–98° and $(\alpha)_D + 50°$ in $CHCl_3$, prepared from (+)-hydratropic acid. (Mislow & Heffler (1952) give m.p. 100–101° and $[\alpha]_D + 59·5°$ in $CHCl_3$ for the (+)-amide, and Wild (1947) gives m.p. 92° for the (±)-amide.) Further proof of the stereochemical nature of the glucuronide was obtained by feeding (+)-hydratropic acid.

From (+)-hydratropic acid. (±)-Hydratropic acid was prepared from the aldehyde and resolved according to Arcus & Kenyon (1939). The (+)-hydratropic acid obtained had $[\alpha]_D + 73°$ in ethanol and thus contained about 95% of the (+)-isomer. (Raper (1923) gives $[\alpha]_D^{20} + 81·1°$ in ethanol.) A total of 2·9 g. of the acid was fed to three rabbits, and the 24 hr. urine worked up as described above. Methyl (+)-hydratropoylglucosiduronate dihydrate, m.p. 166° and $[\alpha]_D + 24°$ in 30% aqueous ethanol (*c*, 0·2), was obtained. (Found: C, 51·6; H, 6·4%). It was identical with the methyl ester isolated after feeding (±)-hydratropic aldehyde.

A partially resolved sample of (−)-hydratropic acid was also prepared. It had $[\alpha]_D^{20} - 44°$ and thus contained about 77% of the (−)-isomer. Rabbits when fed with this compound yielded a highly reducing urine, but the glucuronide gum from the urine could not be induced to crystallize nor could any crystalline derivative be prepared.

Hydratropyl glucosiduronic acid (2-phenyl-n-propyl glucosiduronic acid)

(±)-Hydratropic alcohol could be expected to yield in the rabbit at least four glucuronides, namely, (+)- and (−)-hydratropyl glucosiduronic acids as well as (+)- and (−)-hydratropoylglucosiduronic acids. Both types (i.e. ether and ester) of glucuronide are, in fact, formed, but the amount of one in relation to the other was very variable in different experiments. In most experiments large amounts of hydratropoylglucuronide and only small amounts of hydratropyl glucuronide appeared to be excreted. Occasionally appreciably more of the alcohol glucuronide was formed.

The following experiment gave a relatively good yield of hydratropyl glucuronide. (±)-Hydratropic alcohol (3 g.) was fed to two rabbits. The 24 hr. urine gave an intense naphthoresorcinol reaction and readily reduced Fehling's and Benedict's reagents. It was made 2N with respect to NaOH and kept at 100° for 30 min. in order to hydrolyse the hydratropoylglucuronide. After cooling, the urine was neutralized with glacial acetic acid and then worked up by the lead acetate procedure to yield the glucuronide gum. The latter was dissolved in 2 ml. ethanol and the solution poured into ether (200 ml.) to precipitate any free glucuronic acid. After standing a short while, the ether was filtered and ethereal diazomethane added. After evaporation of the solvents, the residue was acetylated and the product poured into water. The solid (4 g.) which separated was recrystallized twice from ethanol, yielding *methyl (hydratropyl tri-O-acetyl-β-D-glucosid)uronate* (*methyl (2-phenyl-n-propyl tri-O-acetyl-β-D-glucosid)uronate*), as white needles, m.p. 123–124° and $[\alpha]_D^{20} - 35°$ in $CHCl_3$ (*c*, 1). (Found: C, 58·1; H, 6·0. $C_{22}H_{28}O_{10}$ requires C, 58·4; H, 6·2%.) It did not reduce Fehling's or Benedict's reagents.

In another experiment hydrolysis of the ester glucuronide with alkali was omitted. The glucuronide gum was prepared, methylated and acetylated and the hydratropyl glucuronide triacetyl methyl ester, m.p. 123°, isolated as above. The mother liquors on allowing to stand deposited crystals of the reducing methyl (+)-hydratropoyl-tri-*O*-acetylglucosiduronate, which on recrystallization had m.p. 132° and did not depress the m.p. of the (+)-isomer of this triacetyl methyl ester prepared from (+)-hydratropic acid. In two quantitative experiments it was found that the ratio of ester glucuronide to ether glucuronide was roughly 6:1.

2-Phenylisopropyl glucosiduronic acid

A total of 7·75 g. of 2-phenylpropan-2-ol was fed to four rabbits. The neutral 24 hr. urine did not reduce Benedict's or Fehling's solutions, but gave a very rapid and intense naphthoresorcinol reaction. On warming with dilute acid, the urine became cloudy owing to separation of oily droplets. It thus contained a glucuronide labile in dilute acid. Part of the urine was worked up by the usual lead acetate procedure, but it was found that on concentrating the aqueous solution of the glucuronide obtained at the stage of removal of lead by H_2S, the glucuronide decomposed to give an oily emulsion. The rest of the urine was therefore worked up by the usual lead acetate procedure to the stage of removal of lead with H_2S. The aqueous solution thus obtained was not concentrated but extracted continuously with ether. The ether extract, as such, was then methylated with diazomethane and after removal of the ether by evaporation at low temperature the residue was acetylated in the usual way. On pouring the acetylated mixture into water, a

crystalline solid (130 mg.) was obtained which was recrystallized from ethanol. The *methyl (2-phenylisopropyl tri-O-acetyl-β-D-glucosid)uronate* formed colourless needles, m.p. 122–123° and $[\alpha]_D^{20} - 41{\cdot}5°$ in $CHCl_3$ (*c*, 1). (Found: C, 58·6; H, 6·1. $C_{22}H_{28}O_{11}$ requires C, 58·4; H, 6·2%.) The above ester (0·5 g.) was dissolved in a little ethanol and slowly titrated with ethanolic KOH so that the pH remained about 8. On keeping the solution *potassium 2-phenylisopropyl glucosiduronate* (100 mg.) separated as needles. It was recrystallized from ethanol and had m.p. 250° (decomp.) and $[\alpha]_D^{20} - 54°$ in water (*c*, 1·5). (Found: C, 48·4; H, 5·9. $C_{15}H_{19}O_7K$, H_2O requires C, 48·9; H, 5·8%.)

Experiments with cumene

The qualitative reactions of the urine of rabbits receiving cumene have already been described (Smith *et al.* 1954*a*). They indicated the presence of an alkali-labile ester glucuronide, and a glucuronide very sensitive to dilute acid. Using paper chromatography and countercurrent extraction it could be shown that at least three glucuronides were present in the urine.

Chromatography of the urine. A portion of the 24 hr. urine of rabbits which had received 2 ml. each of cumene was subjected to systematic lead acetate precipitation (cf. Kamil *et al.* 1951). The aqueous solution left after removal of the lead from the basic lead acetate precipitate with H_2S was used for chromatography. A spot of this solution was placed on the starting line using Whatman no. 1 paper and *n*-butanol saturated with water used as the solvent. The chromatogram was allowed to run overnight (16 hr.). The position of the glucuronides on the paper was detected by using two sprays separately. The glucuronides as acids were detected by the blue spots given when the paper was sprayed with an aqueous solution containing KI, KIO_3 and starch (1% each, w/v). Glucuronic acid was detected by spraying the paper with 20% (w/v) trichloroacetic acid in *n*-butanol containing 0·1% naphthoresorcinol and then heating the paper at 110° for 15 min. Blue spots develop readily when acid-labile glucuronides were present. The iodide–iodate–starch spray revealed three spots of R_F 0·1–0·15, 0·6–0·7 and 0·9–0·95. The naphthoresorcinol spray also revealed three spots of the same R_F, but whilst the two slower ones were intense, the fast moving spot was weak. This indicated that the fast moving spot was due to a relatively stable glucuronide probably present in small amount.

Countercurrent extraction. An attempt was made to separate these glucuronides on a preparative scale by countercurrent distribution using *n*-butanol and 4% (w/v) NaCl as solvents. In two experiments seven and twelve separating funnels respectively were used. The *n*-butanol layers were analysed spectroscopically for benzene-like absorption at 280 mμ. and the aqueous layers for glucuronic acid by the naphthoresorcinol method. Again three glucuronides were detected. The first was slow moving and tended to remain stationary in the first portion of aqueous solvent. The other two separated in the *n*-butanol. Attempts to isolate the glucuronides by working up the relevant fractions by the lead acetate method failed because of dilution and the presence of chloride, which gave rise eventually to acid conditions sufficient to decompose the glucuronides at the final stage of evaporation. However, sufficient information was obtained by these procedures to allow isolation of each glucuronide in separate experiments. Thus one glucuronide was unstable to alkali, the second was unstable to dilute acid and the third was relatively stable to both dilute acid and alkali.

Isolation of hydratropyl glucuronide. Six rabbits were each fed with 1 ml. of cumene. The 24 hr. urine was made 2N with respect to NaOH and then heated for 5 hr. on a boiling-water bath under reflux to decompose ester glucuronide. The mixture was acidified with HCl and extracted with ether. The ether extract yielded on evaporation 0·3 g. of hydratropic acid ($[\alpha]_D^{20} + 25°$ in ethanol) containing an excess of the (+)-isomer. The residual urine was worked up by the lead acetate procedure to the stage of removal of lead with H_2S. The aqueous filtrate after removal of PbS was continuously extracted with ether for 6 hr. This ether extract was then methylated with diazomethane and the gummy methyl ester acetylated with pyridine and acetic anhydride. On pouring the product into water, 0·12 g. of crude triacetyl methyl ester was obtained. This on recrystallization from ethanol yielded 60 mg. of pure methyl (hydratropyl tri-*O*-acetylglucosid)uronate m.p. 123° and $[\alpha]_D^{20} - 35°$ in $CHCl_3$ (*c*, 1). (Found: C, 58·5; H, 6·2%.) It was non-reducing and did not depress the m.p. of the authentic triacetyl methyl ester prepared biosynthetically from hydratropyl alcohol. It considerably depressed the m.p. of methyl (2-phenyl*iso*propyl tri-*O*-acetyl glucosid)-uronate, m.p. 122–123°.

Isolation of 2-phenylisopropyl glucosiduronic acid. A total of 18 g. of cumene was fed to six rabbits and the 24 hr. urine was worked up by the lead acetate method. The filtrate from PbS was freeze-dried in order to avoid decomposition of the acid-labile glucuronide. The resulting acidic glucuronide gum was taken up in 20 ml. 90% (v/v) aqueous ethanol and a large volume of ether added, sufficient to produce two layers. The top ether layer was methylated with ethereal diazomethane, and the solvent removed and the residue acetylated in the usual manner. On pouring the product into water, 8 g. of crude triacetyl methyl ester were obtained. The urine from the second 24 hr. after feeding was similarly treated and yielded 1 g. of triacetyl methyl ester (total 9 g., equivalent to 15% of the dose). On recrystallization from ethanol, 1·5 g. of pure methyl (2-phenyl*iso*propyl tri-*O*-acetyl glucosid)uronate, m.p. and mixed m.p. 123° and $[\alpha]_D^{20} - 42{\cdot}1°$ in $CHCl_3$ (*c*, 1) was obtained. (Found: C, 57·9; H, 6·0%.)

Isolation of hydratropoylglucuronide. Six rabbits were each fed 2 ml. cumene and the glucuronide gum was prepared from the 24 hr. urine in the usual way. The gum was dissolved in 10 ml. methanol and the solution filtered to remove a small amount of inorganic matter. The methanol solution was poured into 500 ml. ether and a ppt. separated. This ppt. was dissolved in a little aqueous methanol and methylated with ethereal diazomethane. The resulting solution was evaporated under reduced pressure to a syrup, which slowly crystallized. Recrystallization from water yielded the neutral methyl (+)-hydratropoylglucosiduronate (50 mg.), m.p. and mixed m.p. 167° and $[\alpha]_D^{25} + 24°$ in 30% aqueous ethanol (*c*, 0·25). The ester reduced Fehling's solution and was sparingly soluble in water and methanol but more soluble in mixtures of these solvents. On acetylation it yielded methyl (+)-hydratropoyl-tri-*O*-acetyl-glucosiduronate, m.p. and mixed m.p. 132°. (Found: C, 56·4; H, 5·7%.)

The action of dilute acid on cumene urine

Detection of a styrene derivative. When cumene urine is heated with dilute acid, oily drops separate and these were

suspected to contain α-methylstyrene (cf. Smith *et al.* 1954*a*). Styrene and its α- and β-methyl derivatives in ethanol show an absorption band at about 240–250 mμ. with an extinction in the region of 10 000. ($\lambda_{max.}$ for styrene is 244 mμ., for α-methylstyrene, 242 mμ. and for β-methylstyrene 246 mμ.; see Gillam & Stern, 1954; Magat & Maier, 1943.) If a styrene derivative is formed from cumene urine, it could arise from 2-phenylpropan-2-ol or its glucuronide and possibly from 2-phenylpropan-1-ol (hydratropyl alcohol) or its glucuronide. Both these alcohols have only a low absorption in the 240–250 mμ. region (Fig. 1).

Two rabbits were each fed with 2 ml. of cumene and the 24 hr. urine was treated with 0·1 vol. of conc. H_2SO_4. The mixture was heated under reflux for 2 hr. on the water bath and then steam-distilled. The distillate containing oily droplets which gave no colour for phenols with $FeCl_3$ was extracted with ether (200 ml.). The extract was washed with 2N-NaOH (2 × 10 ml.) to remove volatile acids (see below) and, after drying over anhydrous Na_2SO_4, was evaporated to yield a neutral oil (0·9 g. or 26% of the dose on the assumption that the oil is α-methylstyrene). In ethanol the oil showed a large band at 240 mμ. (Table 2) which suggested that it was in part a styrene derivative, although we failed to make a solid derivative in order finally to identify it. It was purified by distillation and had b.p. 169°/760 mm., n_D^{22} 1·5324 and $\lambda_{max.}$ 242–243 mμ. with log ε 4·01 in ethanol; α-methylstyrene had b.p. 161–162°, n_D^{21} 1·5333 and n_D^{24} 1·5311 and $\lambda_{max.}$ 242 mμ., log ε 4·05 (Magat & Maier, 1943). This material appears to arise from 2-phenylpropan-2-ol or its glucuronide, since this alcohol on treatment with dilute acid also yields a neutral oil with a high extinction at 240 mμ. The results of the action of acid and alkali upon the isomeric 2-phenylpropanols are shown in Table 2. It is to be noted that hydratropyl alcohol is relatively stable to acid and alkali.

Isolation of hydratropic acid. The NaOH washings of the ether extract described in the preceding paragraph were acidified and extracted with ether. Evaporation of the ether left a brownish oil (0·49 g. or 14% of the dose) which appeared to be mainly hydratropic acid. After distillation the colourless oil had $[\alpha]_D^{20}$ +25° in ethanol and thus appeared to contain an excess of (+)-isomer. On conversion of the material into the amide a small amount was obtained of what appeared to be (+)-hydratropamide, m.p. 95–97°, which did not depress the m.p. of authentic (+)-hydratropamide, m.p. 97–98°. (Found: C, 72·4; H, 7·6; N, 9·0. Calc. for $C_9H_{11}ON$: C, 72·4; H, 7·4; N, 9·4%.) The amount of the amide obtained was insufficient for a determination of the optical rotation. In another experiment 8 ml. cumene were fed and 300 ml. urine collected. By titration with Benedict's reagent, the urine contained 1 g. of hydratropic acid combined with glucuronic acid. This urine was mixed with 25 ml. concn. H_2SO_4 and heated for 4 hr. It was then extracted with ether in a separating funnel. The acids in the ether extract were transferred to NaOH, the alkaline extract acidified and the acids transferred to ether. The ether was evaporated and the resulting syrup treated with thionyl

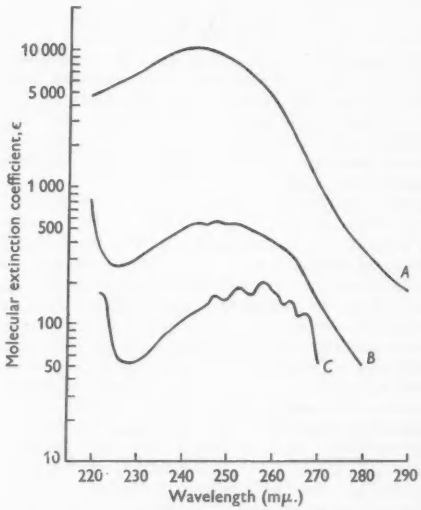


Fig. 1. The ultraviolet absorption spectra of α-methylstyrene (curve *A*), 2-phenylpropan-2-ol (*B*) and 2-phenylpropan-1-ol (hydratropic alcohol; *C*) in ethanol. (α-Methylstyrene, $\lambda_{max.}$ 242–243 mμ., ε 10200; 2-phenylpropan-2-ol, $\lambda_{max.}$ 244, 247·5, 250 mμ., ε 545, 558, 552; 2-phenylpropan-1-ol, $\lambda_{max.}$ 248, 252, 258, 264, 267 mμ., ε 157, 190, 210, 149, 126.)

Table 2. *The effect of acid and alkali upon the isomeric 2-phenylpropanols*

Compound	Treatment	Extinction in ethanol at 240 mμ. ($E_{1\,cm.}^{1\%}$)
α-Methylstyrene	None	835·0
Neutral oil from acid hydrolysis of cumene urine	None (see text)	550·0
2-Phenylpropan-2-ol	None	55·5
	Steam distilled	57·5
	15 min. at 100° in 2N-HCl, then steam distilled	380·0
	3 hr. at 100° in 2·5N-NaOH, then steam distilled	51·5
2-Phenylpropan-1-ol (hydratropic alcohol)	None	11·0
	15 min. at 100° in 2N-HCl, then steam distilled	11·0
	1·5 hr. at 100° and 18 hr. at 20° in 2N-HCl, then steam distilled	19·4

chloride. The product was treated with NH_3 and the solution neutralized and exhaustively extracted with ether. On evaporation of the ether and recrystallization of the residue from *n*-hexane-benzene, hydratropamide (50 mg.), m.p. 91–94° and $(\alpha)_D$ +44° in $CHCl_3$ (*c*, 1), was obtained. (Found: C, 71·7; H, 7·4. Calc. for $C_9H_{11}ON$: C, 72·4; H, 7·4%.) This amide thus contained over 90% of the (+)-isomer, but its m.p. was a little higher than that of the (±)-amide, m.p. 91–92°. Mixtures of the (+) and (±)-amide were found to melt between 91 and 97°. The m.p. of the (±)-amide was not depressed but raised by admixture with the (+)-amide.

Approximate estimation of 2-phenylisopropyl glucosiduronic acid. When potassium 2-phenyl*iso*propyl glucosiduronate is heated with dilute acid it does not yield α-methylstyrene quantitatively. However, using constant conditions it yielded material with a maximum absorption at 244 mμ. in reproducible amounts to within ±10%. A calibration curve for the approximate estimation of this glucuronide was therefore constructed as follows. One ml. of a solution of the potassium salt of the glucuronide (0·05–0·5 mg./ml.) was heated with 1 ml. 2N-H_2SO_4 for 30 min. in a stoppered test tube at 110–120°. After cooling, 1 ml. of 20% (w/v) NaOH was added and the mixture shaken with 10 ml. of *cyclo*hexane (spectroscopic grade). The upper layer was filtered through a cotton wool plug and its optical density measured at 244 mμ. against a *cyclo*hexane blank in a Unicam spectrophotometer SP 500. The same procedure was used for rabbit urine, 1 ml. of urine diluted 25 times being substituted for 1 ml. of glucuronide solution. Recoveries of 0·3 mg. glucuronide added to 1 ml. diluted urine were within ±10%. This procedure was used to obtain the approximate amount of 2-phenylpropan-2-ol and its glucuronide present in cumene urine. In three animals which had each received 0·86 g. (1 ml.) cumene, it was estimated that 37, 38 and 40% of the cumene fed was converted into 2-phenylpropan-2-ol and its glucuronide. In two of these animals the hydratropoylglucuronide output was also estimated by titrating the urine with Benedict's reagent standardized against pure hydratropoylglucuronide. The values found were 24 and 25% of the dose. The total glucuronide excreted by these animals estimated by the naphthoresorcinol method was about 86% of the dose of cumene, so that by difference, i.e. total glucuronide—(hydratropoylglucuronide + 2-phenyl*iso*propyl glucuronide), the hydratropyl glucuronide is roughly 20–25%. By these approximate methods we conclude that cumene yields about 40% of 2-phenylpropan-2-ol and 25% each of hydratropic acid and alcohol.

DISCUSSION

The present work shows that the side chain of cumene (I) is oxidized in the rabbit at the ω and (ω-1) carbon atoms, yielding 2-phenylpropan-2-ol (II), hydratropic alcohol (III) and hydratropic acid (IV):

$$\underset{\text{(I)}}{\text{Ph.CH(CH}_3)_2} \rightarrow \underset{\text{(II)}}{\text{Ph.C(OH)(CH}_3)_2}$$

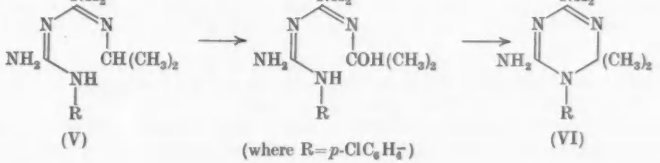
$$+\underset{\text{(III)}}{\text{Ph.CH(CH}_3).\text{CH}_2\text{OH}} \rightarrow \underset{\text{(IV)}}{\text{Ph.CH(CH}_3).\text{COOH}}$$

All three oxidation products have been isolated as glucuronides and thereby characterized. Approximately 40% of the cumene is oxidized to (II) and about 25% to each of (III) and (IV), the total ω-oxidation being about 50%. It appears thus that oxidation takes place more readily at the carbon atom adjacent to the ring than at the ω-carbons since there are two of the latter. It is interesting to note that autoxidations of cumene, which are purely chemical reactions at the methyne carbon atom, proceed by free radical mechanisms, the radical $Ph\dot{C}Me_2$ being formed (cf. Waters, 1945; Melville & Richards, 1954).

The formation of a tertiary alcohol by the oxidation of the methyne carbon atom of an *iso*propyl group at the end of an *iso*amyl group has been reported to occur during the metabolism of 5-*iso*amyl-5-ethylbarbituric acid (Amytal) in the dog. The major metabolite of Amytal is 5-ethyl-5-(3′-hydroxy*iso*amyl)barbituric acid (Maynert, 1952). A similar reaction also occurs in man, for Fieser (1947) has shown that 2-hydroxy-3-*iso*amyl-1:4-naphthoquinone is metabolized to 2-hydroxy-3-(3′-hydroxy*iso*amyl)-1:4-naphthoquinone. We suggest that it is also a possible reaction in the conversion of the antimalarial, proguanil (V) into its active metabolite (VI) (Crowther & Levi, 1953) in man and the rabbit.

The conversion of cumene into hydratropic acid and alcohol involves the production of an asymmetric carbon atom next to the benzene ring. In the case of ethylbenzene (Smith *et al.* 1954*b*) it was shown that the biological oxidation produced both isomers of methylphenylcarbinol and in this case the hydroxylated carbon atom also became the asymmetric one. In the case of cumene the carbon atom hydroxylated is not the one which becomes asymmetric:

$$\text{Ph.CH}_2.\text{CH}_3 \rightarrow \text{Ph.CHOH.CH}_3;$$
$$\text{Ph.CH(CH}_3)_2 \rightarrow \text{Ph.CH(CH}_3).\text{CH}_2\text{OH}$$



Both optical forms of hydratropic acid are formed when cumene is fed to rabbits, since a mixture of unequal quantities of the (+)- and (−)-isomers, with the (+)-isomer predominating, can be isolated from the urine. However, when hydratropoylglucuronide was isolated from cumene urine only the (+)-diastereoisomer was obtained in the crystalline state and this in small yields. Hydratropoylglucuronide is also formed when (±)-hydratropic aldehyde is fed to rabbits, but again only the (+)-diastereoisomer could be isolated in the crystalline state. It thus appears that (−)-hydratropoylglucuronide is difficult to obtain crystalline, although it is highly probable that it is formed, since both (±)- and (−)-hydratropic acids when fed to rabbits give high yields of reducing glucuronide (see Table 1). Kay & Raper (1922) have reported that when (±)-hydratropic acid is fed to dogs, about one-third of the dose is excreted combined with glycine, and here again the urinary product contained an excess of the (+)-isomer.

The glucuronide of hydratropyl alcohol isolated from cumene urine as the triacetyl methyl ester was identical with the glucuronide obtained by feeding (±)-hydratropyl alcohol. However, we have been unable to determine the optical form of this glucuronide which could be a derivative of (+)-, (−)- or (±)-hydratropyl alcohol. The glucuronide was only obtained in small yield and on hydrolysis it gave a liquid which appeared to be hydratropyl alcohol, but no satisfactory solid derivative could be made in quantities sufficient for an optical rotation. The rotation ($[\alpha]_D - 35°$ in $CHCl_3$) of the methyl (hydratropyl tri-*O*-acetylglucosid)uronate suggests that the aglycone is in the (±)-form, since its isomer methyl (2-phenyl*iso*propyl tri-*O*-acetylglucosid)uronate, which contains an optically inactive aglycone, has $[\alpha]_D - 41°$. However, since the optical forms of hydratropyl alcohol have low rotations (approx. ±15°; Cohen, Marshall & Woodman, 1915), the rotations of their glucuronides are not likely to differ by more than about 20° (cf. Kamil, Smith & Williams, 1953) and therefore the optical form of the hydratropyl glucuronide isolated in this work has to be left undecided.

It is to be noted that hydratropyl alcohol is a primary alcohol which is not completely oxidized *in vivo* to the corresponding acid (cf. chlorinated ethanols, Smith & Williams, 1954, and 2:2-dimethyl-2-phenylethanol, Robinson & Williams, 1955).

SUMMARY

1. *iso*Propylbenzene (cumene) when fed to rabbits is oxidized to 2-phenylpropan-2-ol, 2-phenylpropan-1-ol (hydratropyl alcohol) and α-phenylpropionic acid (hydratropic acid), which are excreted as conjugated glucuronic acids.

2. The conjugated glucuronic acids of cumene urine have been isolated and characterized as triacetyl methyl esters.

3. The feeding of (±)-hydratropic aldehyde to rabbits results in the excretion of hydratropoylglucosiduronic acid, but only the (+)-diastereoisomer could be isolated in crystalline form. This was identical with the reducing glucuronide isolated after feeding (+)-hydratropic acid or cumene.

4. Hydratropyl alcohol gives rise in rabbits to hydratropyl and hydratropoylglucuronides. The former, characterized as its triacetyl methyl ester, was identical with the non-reducing relatively stable glucuronide found in cumene urine. Only the (+)-diastereoisomer of hydratropoyl glucuronide was isolated.

5. 2-Phenylpropan-2-ol gives rise in rabbits to 2-phenyl*iso*propyl glucuronide, which was isolated and characterized as the triacetyl methyl ester and the potassium salt. This glucuronide is readily decomposed by dilute acid to yield α-methylstyrene and is identical with acid-labile glucuronide of cumene urine.

6. These results are discussed, and it appears that the α-carbon atom of cumene is more readily oxidized *in vivo* than the β-carbons, since the yield of 2-phenylpropan-2-ol is about 40% of the dose, whereas the yields of hydratropyl alcohol and hydratropic acid are 25% each, the total ω-oxidation being about 50%.

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Studies in Detoxication

61. THE METABOLISM OF ALKYLBENZENES. *TERT.*-BUTYLBENZENE

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In earlier papers in this series (Smith, Smithies & Williams, 1954*b*; Robinson, Smith & Williams, 1955) it was shown that ethylbenzene and *iso*propylbenzene were oxidized in rabbits at the activated carbon atom next to the benzene ring. In *tert.*-butylbenzene (I) the carbon atom next to the benzene ring carries no replaceable hydrogen; furthermore, unlike ethyl and *iso*propyl-benzene, it is resistant to autoxidation and is relatively stable to oxidation. *tert.*-Butylbenzene is nevertheless extensively metabolized by rabbits, for Smith, Smithies & Williams (1954*a*) have shown that it causes the excretion of conjugated glucuronic acid corresponding to more than two-thirds of the dose. *tert.*-Butylbenzene could undergo oxidation of its benzene ring or of its methyl groups. Nuclear oxidation would yield a phenol, which would be expected to be excreted in part as an ethereal sulphate. However, very little if any ethereal sulphate is excreted by rabbits receiving *tert.*-butylbenzene (cf. Smith *et al.* 1954*a*). Oxidation of one of the methyl groups might be expected to yield 2:2-dimethyl-2-phenylethanol (II), the corresponding aldehyde (III) or acid (IV).

$$\underset{\text{(I)}}{\mathrm{Ph.CMe_3}} \rightarrow \underset{\text{(II)}}{\mathrm{Ph.CMe_2.CH_2OH}} \rightarrow \underset{\text{(III)}}{\mathrm{Ph.CMe_2.CHO}} \rightarrow \underset{\text{(IV)}}{\mathrm{Ph.CMe_2.COOH}}.$$

Dimethylphenylacetic acid would be expected to yield a reducing ester glucuronide, but the urine from *tert.*-butylbenzene is non-reducing (Smith *et al.* 1954*a*). Dimethylphenylethanol, on the other hand, would yield a non-reducing glucuronide. It will be shown here that 2:2-dimethyl-2-phenylethyl glucuronide appears to be the major if not the only metabolite of *tert.*-butylbenzene in the rabbit.

EXPERIMENTAL

Materials

tert.-Butylbenzene (British Drug Houses Ltd.) was redistilled, b.p. 166–167°.

αα-Dimethyl-α-phenylacetic acid. Phenylacetonitrile (46·8 g.; Light and Co.) was dissolved in liquid NH_3 (300 ml.) containing 50 mg. anhydrous $FeCl_3$ and 32 g. of potassium was added in small pieces to the solution. During the addition of the potassium the solution turned first blue then slowly brownish red. When the potassium had dissolved, slightly more than the calculated amount of methyl iodide (125 g.) was added with shaking and the solution was then allowed to stand until the NH_3 had evaporated. The dark brown oily residue was washed with very dil. acid which contained sodium thiosulphate to remove I_2. The oil was then taken up in ether and the solution dried over anhydrous Na_2SO_4. After evaporation of the ether, the oily residue of dimethylphenylacetonitrile was distilled under reduced pressure (yield 40 g.). The nitrile was mixed with 50 ml. of 80% (v/v) H_2SO_4 and heated under reflux for 3 hr. The product was poured on 200 g. ice and the mixture kept overnight. The solid dimethylphenylacetamide was collected, washed with saturated $NaHCO_3$ and recrystallized repeatedly from 50% aqueous ethanol (m.p. 160°; Haller & Bauer (1912) give m.p. 160°). The amide (20 g.) was dissolved in 80% (v/v) H_2SO_4 and the solution cooled in ice. The calculated amount of a 10% solution of $NaNO_2$ was carefully added below the surface of the solution, and the mixture then kept overnight at room temperature. On pouring on to ice, dimethylphenylacetic acid separated and was collected. It was dissolved in 2N-NaOH and the solution extracted with ether to remove impurities. It was then precipitated by acidification and recrystallized from water (yield 12·5 g., m.p. 76°; Haller & Bauer (1912) give m.p. 80–81°).

2:2-Dimethyl-2-phenylethanol. A solution of the above acid (12·5 g.) in 200 ml. dry ether was run slowly (0·5 hr.) into a suspension of lithium aluminium hydride (4·5 g.) in 250 ml. dry ether, the flask, which was fitted with a condenser, being kept in an ice bath. The mixture was allowed to

warm up to room temp. and then refluxed for 0·5 hr. After keeping overnight, water was carefully added to decompose excess hydride, and the white ppt. which formed was redissolved by adding 10% (v/v) H_2SO_4. The ether layer was now separated and the aqueous layer extracted once with ether. The ether solutions were pooled, washed with 20% (w/v) NaOH and then with water, and finally dried over anhydrous Na_2SO_4. After evaporation of the ether, the residue was distilled under reduced pressure to yield 12 g. of dimethylphenylethanol, b.p. 125° (at 14–20 mm.). The alcohol was characterized as the α-naphthylurethane, m.p. 92° (Whitmore, Weisgerber & Shabica (1943) give m.p. 91·5–92·5°.)

Methods

The glucuronic acid output of rabbits which had received oral doses of *tert.*-butylbenzene and 2:2-dimethyl-2-phenylethanol was determined by the naphthoresorcinol method as modified by Paul (1951) (for results see Table 1).

Isolation of metabolites

From 2:2-dimethyl-2-phenylethanol. A rabbit was given 1 g. of the alcohol by mouth. The 24 hr. urine was neutral and non-reducing, but gave an intense naphthoresorcinol reaction. It gave no colour with $FeCl_3$. The glucuronide gum (1·5 g.), prepared by the basic lead acetate method, was soluble in methanol. On methylation and acetylation (cf. Kamil, Smith & Williams, 1951) it yielded 2 g. of triacetyl methyl ester, which on recrystallization from ethanol followed by *iso*propyl ether yielded colourless needles (0·5 g.) of *methyl (2:2-dimethyl-2-phenylethyl tri-O-acetyl-β-D-glucosid)uronate* m.p. 125°, $[\alpha]_D^{20}$ – 30° in $CHCl_3$ (*c*, 1). (Found: C, 59·4; H, 6·7; OMe, 6·3. $C_{23}H_{30}O_{10}$ requires C, 59·2; H, 6·5; OMe, 6·65%.)

The above ester (0·7 g.) was deacetylated by quickly heating to boiling with 2·5N-NaOH. The solution was cooled and made 2N with respect to H_2SO_4 and heated under reflux for 3 hr. After cooling, the solution was extracted twice with 20 ml. of light petroleum (b.p. 120°). The extract was dried over anhydrous Na_2SO_4 and then refluxed with α-naphthyl*iso*cyanate (200 mg.) for 3 hr. After filtering and cooling, 2:2-dimethyl-2-phenylethyl-α-naphthylurethane separated (200 mg., 45%). This was recrystallized from light petroleum (b.p. 120°) and had m.p. 90°, and mixed m.p. 90–91° with the authentic naphthylurethane.

From αα-dimethylphenylacetic acid. This acid (1 g.) was fed to a rabbit and the 24 hr. urine collected. The urine readily reduced Fehling's and Benedict's solutions and gave a rapid, strong, positive naphthoresorcinol test characteristic of ester glucuronides. The glucuronide was obtained as a reducing gum (1 g.) which on methylation and acetylation failed to yield a crystalline product. On hydrolysis the gum yielded αα-dimethylphenylacetic acid, m.p. and mixed m.p. 76°, showing that this acid had formed an ester glucuronide.

From tert.-Butylbenzene. A total of 2·2 g. of the hydrocarbon was fed to three rabbits and the urine collected for 24 hr. The urine was neutral in reaction, non-reducing and gave no colour reaction with $FeCl_3$. It gave no reaction with 2:4-dinitrophenylhydrazine (Brady's reagent), showing the absence of aldehydes. The glucuronide gum (2 g.) was prepared in the usual manner and was converted into the triacetyl methyl ester. After recrystallization 0·8 g. of pure methyl (2:2-dimethyl-2-phenylethyl tri-*O*-acetyl-β-D-glucosid)uronate, m.p. 126–127° and $[\alpha]_D^{20}$ – 28° in $CHCl_3$ (*c*, 1) was obtained. (Found: C, 58·9; H, 6·3. $C_{23}H_{30}O_{10}$ requires C, 59·2; H, 6·5%.) It was identical in all respects with the ester (mixed m.p. 126°) isolated after feeding 2:2-dimethyl-2-phenylethanol.

The high conjugation of dimethylphenylethanol and of *tert.*-butylbenzene (Table 1), together with the absence of any reducing power in the urine, suggests that only the glucuronide of 2:2-dimethyl-2-phenylethanol is excreted after feeding these compounds and that the αα-dimethylphenylacetylglucuronide is not formed. However, this does not eliminate αα-dimethylphenylacetic acid as a minor metabolite of *tert.*-butylbenzene. This acid could be formed and excreted as such or as a glycine conjugate. The basic lead acetate filtrate obtained during the preparation of the glucuronide from *tert.*-butylbenzene was therefore investigated by paper chromatography. When a spot of this filtrate was chromatographed on Whatman no. 1 paper using saturated *n*-butanol–water as solvent, three acid spots were detected on spraying the paper with iodide–iodate mixtures followed by starch solution. These spots had R_F values of 0·25, 0·6 and 0·9. That the second spot (R_F 0·6) was a glucuronide was revealed by spraying with naphthoresorcinol and heating. This was probably the dimethylphenylethyl glucuronide. The last spot, which was weak, corresponded to αα-dimethylphenylacetic acid which has an R_F of 0·9 under similar conditions. This spot increased in intensity if the filtrate used was subjected to acid hydrolysis before chromatography, suggesting the presence of the glycine conjugate of dimethylphenylacetic acid. Since this acid appeared to be a very minor metabolite, its further identification was not pursued. The spot of R_F 0·25 had a long tail and appeared to be due to traces of free glucuronic acid and glucurone.

DISCUSSION

The present paper completes the study of the major metabolites formed in rabbits from alkylbenzenes containing a linear chain of two carbon atoms. The

Table 1. *Glucuronic acid conjugation of 2:2-dimethyl-2-phenylethanol and tert.-butylbenzene in rabbits*

Results on individual animals. Figures in parentheses are average values. The excretion of extra glucuronic acid was complete in one day after dosing.

Compound fed	Dose (mg./kg.)	Percentage of dose excreted as glucuronide
tert.-Butylbenzene	268 (2 m-moles)	63, 85, 94 (81)
	500 (4 m-moles)	(66)*
2:2-Dimethyl-2-phenylethanol	300 (2 m-moles)	93, 94 (93·5)
	150 (1 m-mole)	92, 93, 100 (95)

* Quoted from Smith *et al.* (1954*a*)

Table 2. *The oxidation products of ethyl-, isopropyl- and tert.-butyl-benzene in rabbits*

Compound	Known oxidation products (% of dose)		
$Ph.CH_2.CH_3$	(±)$Ph.CHOH.CH_3$ (32%)	$Ph.CHOH.COOH$ (1–2%)*	$Ph.COOH$ (30%)†
$Ph.CH(CH_3)_2$	$Ph.COH(CH_3)_2$ (40%)	$Ph.CH(CH_3).CH_2OH$‡ (25%)	$Ph.CH(CH_3).COOH$‡ (25%)
$Ph.C(CH_3)_3$	$Ph.C(CH_3)_2.CH_2OH$ (95%)	$Ph.C(CH_3)_2.COOH$ (traces)	—

* Data from Thierfelder & Daiber (1923).
† Unpublished data (el Masry, Smith & Williams); cf. Thierfelder & Daiber (1923) who give 25%.
‡ Probably both optical forms (cf. Robinson *et al.* 1955).

results are summarized in Table 2 from which it is clear that oxidation at the activated carbon atom next to the benzene ring takes place more readily than at the ω-carbon atom when such an oxidation is possible. In *tert.*-butylbenzene, ω-oxidation is the only possible reaction, and it is to be noted that this oxidation hardly goes beyond the primary alcohol stage. 2:2-Dimethyl-2-phenylethanol ($Ph.C(CH_3)_2.CH_2OH$) contains a primary alcohol group which is not readily oxidized *in vivo* and it should be compared with 2-phenylethanol ($Ph.CH_2.CH_2OH$) and 2-methyl-2-phenylethanol (hydratropyl alcohol, $Ph.CH(CH_3).CH_2OH$). 2-Phenylethanol is almost completely oxidized in the rabbit to phenylacetic acid (Smith *et al.* 1954*b*). 2-Methyl-2-phenylethanol is less readily oxidized, for it is excreted partly as the glucuronide of the unchanged alcohol and partly as α-methylphenylacetic acid (hydratropic acid) (Robinson *et al.* 1955). It appears, therefore, that the substitution of two methyl groups on the β-carbon atom of 2-phenylethanol almost completely inhibits the oxidation of the primary alcohol group *in vivo*. It is thus to be compared with di- and tri-chloroethanols, which are not readily oxidized in the rabbit (Smith & Williams, 1954).

SUMMARY

1. *tert.*-Butylbenzene is oxidized in rabbits mainly to 2:2-dimethyl-2-phenylethanol (up to 90% of the dose), which is excreted as a glucuronide, characterized as a triacetyl methyl ester. Traces of αα-dimethylphenylacetic acid may also be formed.

2. 2:2-Dimethyl-2-phenylethanol is not oxidized in rabbits to any appreciable extent, and 90–100% of doses of 150–300 mg./kg. is excreted as 2:2-dimethyl-2-phenylethyl glucosiduronic acid, which is identical with the glucosiduronic acid excreted after feeding *tert.*-butylbenzene.

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Kinetic Studies on the Metabolism of Foreign Organic Compounds

6. REACTIONS OF SOME NUCLEAR-SUBSTITUTED BENZOIC ACIDS, BENZAMIDES AND TOLUENES IN THE RABBIT

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The previous papers of this series (Bray, Thorpe & White, 1951, 1952*e*; Bray, Humphris, Thorpe, White & Wood, 1952*b*, *c*, *d*) reported a study of the kinetics *in vivo* of some reactions involving benzoic acid and some of its precursors, certain precursors of phenols and a series of substituted phenols. A mathematical model was worked out which could account for the effect upon metabolic fate of dose level, nuclear substituents, quantitative differences between the metabolism of precursors and their conversion products, and species differences. These investigations have now been extended by studying the metabolism in the rabbit of various nuclear substituted toluenes, benzamides and benzoic acids. It has been found that the metabolic processes involved follow kinetics similar to those already reported and values are given for the appropriate velocity constants. There appeared to be no correlation between these values and the size or electronic nature of the substituents.

MATERIALS AND METHODS

Materials. *p*-Acetamidobenzoic acid was prepared by the acetylation of *p*-aminobenzoic acid with acetic anhydride and *p*-methoxytoluene by methylation of *p*-cresol using methyl sulphate and NaOH. *p*-Cyanobenzamide was prepared by treatment of methyl *p*-cyanobenzoate with NH_3 in ethanol at 100° for 8 hr. in a sealed tube. *p*-Nitro-, *p*-chloro-, *p*-fluoro- and *p*-methoxy-benzamides were obtained by the action of aqueous NH_3 (sp.gr. 0·88) on the corresponding acid chlorides, prepared by the action of PCl_5 on the appropriate acids. *p*-Toluamide was prepared by the action of H_2O_2 on *p*-tolunitrile (Noller, 1933) and *p*-cyanobenzoic acid by a Sandmeyer reaction on *p*-aminobenzoic acid. The other compounds investigated were purchased. Melting points and properties of all compounds agreed with those described in the literature.

Methods. The plan of the experiments, the collection of urine samples and the analytical methods were as described in earlier papers (Bray *et al.* 1951, 1952*b*, *c*, *d*). Glycine conjugates were estimated by the method of Bray, Clowes, Thorpe, White & Wood (1952*a*). The velocity constants were calculated by graphical methods. Both 'log' and 'tangent' methods were used as convenient: when both were applied to the same results values in good agreement were obtained.

Symbols. First-order velocity constants for the formation of hippuric acids and of glucuronic acids are denoted by k_h and k_g respectively and those for the conversion of benzamide and of toluene into benzoic acid by $k_{b,\,am.}$ and $k_{b,\,tol.}$; the zero-order velocity constant for hippuric acid formation is denoted by v_h. The symbol, k_s, used in the discussion, refers to the first-order velocity constant for conjugation of a phenol with sulphuric acid.

RESULTS

The analytical results and the values of the velocity constants obtained are summarized in Tables 1 and 2.

Glycine conjugation. It has already been shown (Bray *et al.* 1951) that, at dose levels of 0·3–1·3 g/kg., the rate of conjugation of benzoic acid with glycine was invariably independent of the body level. From the present results it appears that the conjugation of substituted benzoic acids having values for k_h greater than 0·2 hr.$^{-1}$ follows the kinetics of a zero-order reaction unless glycine is administered simultaneously, when the conjugation follows the kinetics of a first-order reaction. This justifies the grouping together of glycine and sulphate conjugation as processes for which there is a critical body level at which the kinetics change from those of a zero to those of a first-order reaction (Bray *et al.* 1952*e*). An example of a typical first-order curve for the conjugation of *p*-toluic acid with glycine is given in Fig. 1; in this experiment glycine was administered simultaneously with the dose. When the same dose of *p*-toluic acid was administered without glycine, the excretion curve for conjugated glycine was linear, as was found (Bray *et al.* 1951) in all experiments with unsubstituted benzoic acid even when glycine was administered with it. The values of v_h are different for different compounds and all are smaller than the average found for benzoic acid itself.

Glucuronic acid conjugation. In every case the glucuronic acid conjugation followed first-order reaction kinetics. Most of the values for k_g are of the same order as that for benzoic acid (0·08 hr.$^{-1}$), except that for *p*-methoxybenzoic acid (0·22 hr.$^{-1}$) which is appreciably greater. It should, perhaps, be mentioned that in some of these experiments the percentage of the dose conjugated with glucuronic

Table 1. *Velocity constants for glycine and glucuronic acid conjugation of p-substituted benzoic acids by the rabbit*

Dose level 0·4 g./kg. Where values for both zero- and first-order velocity constants are given for the same experiment, the former is evaluated from the first part of the curve and the latter from the last part. Values in italics are for experiments in which 3–4 equivalents of glycine were given with the dose. The percentages recorded under 'excreted unconjugated' are the differences between those for the total ether-soluble acid and the ether-soluble conjugates, i.e. glycine conjugates + ether-soluble glucuronic acid conjugates. (The glucuronic acid conjugates of the halogenobenzoic acids and of *p*-toluic acid are ether-soluble.) The means are geometric. Values (mean) for benzoic acid are from Bray *et al.* (1951).

Substituent	Conjugation with glycine (% of dose)	v_h (mg. glycine/hr.)	k_h (hr.$^{-1}$)	Conjugation with glucuronic acid (% of dose)	k_g (hr.$^{-1}$)	Excreted unconjugated (% of dose)	Rate of linear excretion of unconjugated acid (mg./hr.)
None	83	163	—	15	0·08	1	—
p-CN	35	—	0·17	0	—	68	—
	26	—	0·23	2	0·013	78	—
	33	—	*0·13*	*2*	*0·008*	*53*	—
	46	—	*0·23*	*3*	*0·014*	*65*	—
Mean	—	—	**0·19**	—	**0·011**	—	—
p-NO_2	6	—	0·04	6	0·04	70	—
	8	—	0·03	6	0·02	88	—
	20	—	*0·09*	6	*0·02*	*78*	—
	13	—	*0·06*	1	*0·04*	*80*	—
Mean	—	—	**0·05**	—	**0·03**	—	—
p-OCH_3	39	30	—	59	0·17	0	—
	33	36	—	59	0·36	0	—
	41	31	—	*52*	0·29	3	—
	50	—	*0·21*	*43*	*0·20*	*8*	—
	52	—	*0·16*	*40*	*0·13*	*0*	—
Mean	—	—	**0·18**	—	**0·22**	—	—
p-I	35	17	—	53	0·14	12	—
	46	20	—	39	0·09	0	—
	77	—	*1·2*	*9*	*0·03*	3	—
	65	*16*	—	*12*	—	0	—
	62	—	*1·1*	*18*	*0·18*	0	—
Mean	—	—	**1·1**	—	**0·09**	—	—
p-Cl	83	91	0·38	9	0·04	8	—
	78	107	—	3	—	4	—
	88	—	*0·45*	*3*	*0·02*	*4*	—
	82	—	*0·39*	*4*	*0·02*	*11*	—
	91	—	*0·36*	*5*	*0·02*	*8*	—
Mean	—	—	**0·39**	—	**0·02**	—	—
p-F	83	108	1·2	7	0·10	5	—
	84	—	*1·3*	*4*	*0·06*	*8*	—
	88	—	*0·88*	*2*	*0·02*	*6*	—
	68	—	*1·22*	*2*	*0·04*	*25*	—
	80	—	*1·0*	*4*	*0·04*	*9*	—
	76	—	*1·04*	*2*	*0·03*	*12*	—
Mean	—	—	**1·1**	—	**0·04**	—	—
p-CH_3	47	55	—	17	0·06	46	—
	57	55	—	23	—	30	—
	68	—	*0·43*	*8*	*0·05*	*34*	—
	76	—	*0·37*	*12*	*0·07*	*13*	—
Mean	—	—	**0·4**	—	**0·06**	—	—
p-$NHCOCH_3$	2	—	0·01*	0	0·002*	77	125
	10	—		*1*		*92*	*100*
	7	—		*1*		*82*	*90*
	3	—		*1*		*89*	*100*

* Excretion of conjugates too small for accurate evaluation.

acid and, therefore, the actual amount of glucuronic acid determined was very small. These values may be subject to a greater experimental error than those for experiments in which a larger percentage of the dose was excreted as glucuronide.

Hydrolysis of amides. The results in Table 2 show that the hydrolysis of all the amides studied followed first-order kinetics with respect to the body level of amide. The values obtained for the velocity constant ranged from 0·7 hr.$^{-1}$ for *p*-fluorobenzamide to 0·23 hr.$^{-1}$ for *p*-nitrobenzamide, as compared with 0·32 hr.$^{-1}$ for benzamide. *In vitro*, however, *p*-nitrobenzamide has always been more readily hydrolysed than other amides (e.g. Bray, James, Thorpe & Wasdell, 1950*a*).

Oxidation of toluenes. This process also followed first-order reaction kinetics for all the compounds examined (Table 2).

DISCUSSION

In studies of the metabolism of foreign organic compounds satisfactory interpretation of analyses of 24 hr. urines is difficult (see Bray *et al.* 1952*e*), and kinetic studies seem to offer a more promising method of investigation. A knowledge of the type of kinetics involved assists the interpretation of the variation of metabolic fate with varying dose level;

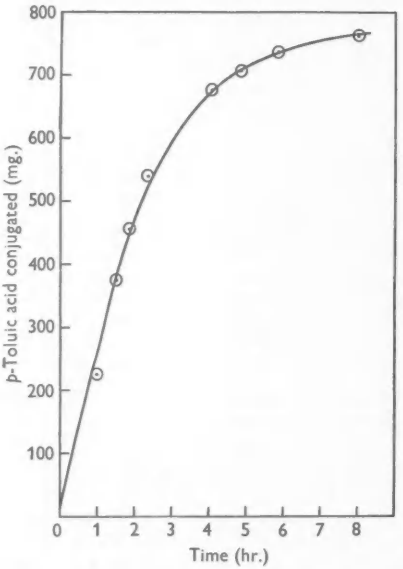

Fig. 1. Excretion curve for the conjugate of *p*-toluic acid with glycine in the rabbit. Glycine (4 g.) was administered with *p*-toluic acid (1 g.). The curve is the theoretical one for $k_h = 0{\cdot}37$ and the points those obtained by experiment.

the variation in the metabolic fate of different nuclear-substituted derivatives depends on the numerical value of the velocity constants. Further, there is the possibility that in the numerical values of velocity constants there may be regularities which can be correlated with other known properties; such observations might permit the prediction of metabolic fates. For example, it has already been shown (Bray *et al.* 1952*c*), that there is a correlation between the rate of glucuronic acid conjugation of phenols and the size of the substituent in the *para* position.

The velocity constants for the formation of ester glucuronides from substituted benzoic acids show no correlation with size or electronic influence of the substituent group. Quick's (1932) generalization, relating to the dog, that 'there is a fairly constant, though not absolute, correlation between the pK of an acid and the rate with which it is excreted combined with glycuronic acid' is not supported by our measurements of k_g values in the rabbit. It is, however, likely that the k_g values for a given acid would be different for the dog and for the rabbit so that a comparison between different species is not valid (see Bray *et al.* 1952*e*).

Quick (1932) noted that in the dog, *para* substitution of benzoic acid had comparatively little effect on the percentage of a dose excreted as glycine conjugate. In the rabbit there is considerable variation in the percentage of the dose excreted in this form, e.g. from 6 % for *p*-acetamidobenzoic acid to 84 % for *p*-chlorobenzoic acid, which is comparable with 83 % for unsubstituted benzoic acid. Without exception, the *para* substituents which we have studied diminish the velocity constant k_h. Although there is no reason to believe that the amount of glycine potentially available in the body is less than that when unsubstituted benzoic acid is conjugated, the values of v_h for *para* substituted benzoic acids are smaller. The reason for this is unknown but two possibilities may be mentioned. Either substituted benzoic acids may inhibit the mobilization of glycine for this purpose or it may be that the first- and zero-order kinetics measured in these experiments are in fact related to Michaelis-type kinetics of an enzyme or enzymes concerned in the metabolic process. Thus the zero-order velocity constant may correspond to the 'maximum velocity' and the first-order constant directly to that of Michaelis kinetics. Hearon (1952) has pointed out that, even in multienzyme systems in the steady state, variations in the overall rate, which occur when the concentration of only one substrate is varied, will follow Michaelis kinetics.

It should be noted that our interpretation of the results is based upon the assumption that the transition from zero- to first-order kinetics as body level falls is abrupt and that intermediate stages

which have fractional order kinetics are ignored (cf. Bray *et al.* 1951). In the earlier experiments the curves obtained were either almost entirely linear or entirely exponential. In the present work, however, some curves were obtained which had both linear and exponential portions of such length that both zero- and first-order velocity constants could be evaluated in a single experiment. An example of this transition from zero- to first-order kinetics is given in Fig. 2, which also shows linear and exponential curves of the type more usually obtained. Curves *A* and *B* are entirely exponential from the time at which absorption can be assumed to be complete. Curve *D* is linear until excretion is practically complete, or, if a transition does occur, it occupies so short a time that the shape of the curve cannot be accurately determined. Curve *C* is linear up to 3 hr. but after that it is exponential.

Table 2. *Velocity constants for hydrolysis of* p*-substituted benzamides and oxidation of* p*-substituted toluenes in the rabbit*

Dose level 0·3 g./kg. Values in italics are for experiments in which 3–4 equivalents of glycine were given with the dose. The percentages recorded under 'total excreted as acid' are the sum of those for the total ether-soluble acid + acid conjugated with glucuronic acid unless glucuronic acid conjugates are ether-soluble (see Table 1). Means are geometric. Values (means) for benzamide and toluene from Bray *et al.* (1951).

	Benzamides			Toluenes		
Substituent	Total excreted as acid (% of dose)	Conjugation as ester glucuronide (% of dose)	$k_{b,\,ben.}$ (hr.$^{-1}$)	Total excreted as acid (% of dose)	Conjugation as ester glucuronide (% of dose)	$k_{b,\,tol.}$ (hr.$^{-1}$)
None	—	—	**0·32**	—	—	**0·11**
p-CN	86	1	0·35	91	1	0·34
	82	1	0·33	*92*	1	*0·28*
	85	1	0·28	105	1	0·32
Mean	—	—	**0·32**	—	—	**0·31**
p-NO_2	*90*	1	*0·27*	32*	1	0·04
	79	1	0·19	27*	1	0·08
Mean	—	—	**0·23**	—	—	**0·06**
p-OCH_3	62	18	0·27	*45*†	2	*0·09*
	64	*13*	*0·28*	*48*†	*2*	*0·12*
	65	*10*	*0·28*	58†	5	0·14
Mean	—	—	**0·28**	—	—	**0·12**
p-Cl	94	1	0·68	83	1	0·26
	92	1	0·50	72	1	0·14
	104	1	*0·53*	64	1	0·11
	97	1	*0·42*	65	1	0·10
Mean	—	—	**0·53**	—	—	**0·14**
p-F	*96*	1	*0·61*	69	1	0·21
	95	1	*0·70*	55	1	0·19
	96	1	*0·81*	64	1	0·14
Mean	—	—	**0·70**	—	—	**0·18**
p-CH_3	*95*	1	*0·53*	62	1	0·11
	92	1	0·37	83	1	0·15
	88	1	*0·41*	80	1	0·17
	81	1	0·41	—	—	—
	92	1	0·32	—	—	—
Mean	—	—	**0·40**	—	—	**0·14**
p-$NHCOCH_3$	—	—	—	92	8	0·63
	—	—	—	91	8	0·56
Mean	—	—	—	—	—	**0·59**
p-SO_2NH_2	—	—	—	*97*	1	*0·25*
	—	—	—	93	1	0·25
Mean	—	—	—	—	—	**0·25**

* Remainder of dose not accounted for.

† Remainder of dose mainly excreted as conjugates of *p*-cresol (Bray, Craddock & Thorpe, unpublished).

Where glycine availability is a limiting factor the actual shape of the excretion curve will depend on the relative body levels of glycine and conjugating acid at any given moment. In the experiment which provided curve *C* presumably the availability of glycine was the limiting factor during the first 3 hr. but after this time the body level of *p*-fluorobenzoic acid had diminished (60 % of the dose had already been excreted) to such an extent that the availability of glycine was then no longer a limiting factor. In all the experiments in which glycine was administered simultaneously with the dose this limiting factor was eliminated with the exception of one experiment with *p*-iodobenzoic acid (see Table 1).

The results for the hydrolysis of *para* substituted benzamides are, in general, such as might have been expected from results previously obtained *in vivo* and *in vitro* with liver extracts or slices (e.g. Bray *et al.* 1950*a*). There appears to be no correlation between the extent of amide hydrolysis and the electronic nature or size of the substituent group. The $k_{b,\ am.}$ values for amides substituted with nitro, cyano and chloro groups, which are strongly deactivating in the *para* position, are of the same order as those for amides substituted with methoxy or methyl groups, which are activating in the *para* position. It can be calculated from the k_g and k_s values (Bray *et al.* 1952*c*, *d*) and the 24 hr. excretion

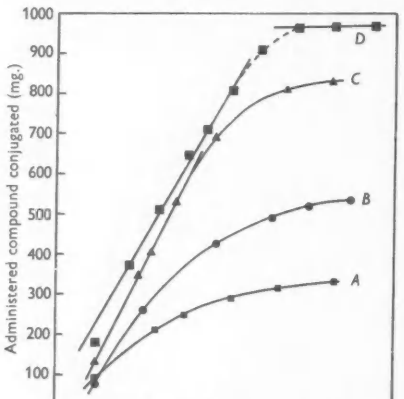

Fig. 2. Typical curves for excretion of glycine conjugates. The compound and the dose administered to the rabbit are given in parentheses. *A*, exponential curve $k_h = 0{\cdot}13$ (*p*-cyanobenzoic acid, 1 g. + glycine, 4 g.); *B*, exponential curve $k_h = 0{\cdot}21$ (anisic acid, 1 g. + glycine, 4 g.); *C*, a curve which is initially linear but after 3 hr. is exponential, $k_h = 1{\cdot}2$ (*p*-fluorobenzoic acid, 1 g.); *D*, a curve which is linear until excretion is almost complete (*p*-fluorobenzoic acid, 1 g.).

(Bray, Ryman & Thorpe, 1947) that *p*-hydroxybenzamide, in which the hydroxyl group is strongly activating, has the very low $k_{b,\ am.}$ value of 0·05 hr.$^{-1}$. The very small extent of hydrolysis of this amide made a direct determination of the velocity constant impracticable. Another amide with a strongly activating substituent, *p*-aminobenzamide, could not be examined experimentally for the same reason; this amide is extensively metabolized by conjugation with acetic acid through the amino group (Bray, Lake, Neale, Thorpe & Wood, 1948).

The values for the velocity constants for the oxidation of substituted toluenes to the corresponding benzoic acids show that there is no correlation between the extent of the oxidation and the nature of the substituent group. The velocity constant for *p*-acetotoluidide is conspicuously high. All the values of $k_{b,\ tol.}$ are higher than that for toluene itself with the exception of that for *p*-nitrotoluene. With this compound, however, only about 30 % of the dose was accounted for. With *p*-methoxytoluene, in experiments without added glycine, about 25 % of the dose was excreted as conjugates of phenols, mainly *p*-cresol (Bray, Craddock & Thorpe, unpublished). The value of $k_{b,\ tol.}$ for *p*-cresol, calculated from 24 hr. values (Bray, Thorpe & White, 1950*b*) and assuming k_g and k_s values are the same as those of phenol, is 0·08 hr.$^{-1}$.

From the compounds which have been examined in this investigation it would appear that the effect of *para* substituents upon the velocity constants for reactions involving the formation or conjugation of benzoic acids is not related to the size or electronic influence of the substituent group. This is in contrast to the effect of *para* substituents upon the velocity constants for the conjugation of phenols with glucuronic acid where it was found that small substituents had no significant effect on the value of k_g but large groups diminished this value.

SUMMARY

1. The kinetics of the conjugation of various *para* substituted benzoic acids with glycine and glucuronic acid, the oxidation of *para* substituted toluenes and the hydrolysis of *para* substituted benzamides have been investigated in the rabbit.

2. All the processes studied followed first-order reaction kinetics with respect to the administered compound except the glycine conjugation of *p*-methoxy-, *p*-iodo-, *p*-chloro-, *p*-fluoro- and *p*-methyl-benzoic acids, administered as such under conditions of glycine inadequacy, when zero-order kinetics were followed.

3. Values for the velocity constants are given.

4. No correlation could be found between the magnitude of the velocity constants and the electronic nature or the size of the substituent groups.

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The Hydrolysis of *n*-Hexanamide by Rabbit-liver Extracts

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Previous papers from this laboratory (Bray, James, Ryman & Thorpe, 1948; Bray, James, Raffan, Ryman & Thorpe, 1949*a*; Bray, James, Thorpe, Wasdell & Wood, 1949*b*; Bray, James, Thorpe & Wasdell, 1950) have given a general account of the properties of an enzyme of rabbit liver which can hydrolyse the carbamoyl groups of a number of aliphatic and aromatic amides. This amidase appeared to be different from the more specific amidases (e.g. glutaminase), and results obtained *in vitro* were in many cases comparable with those found when the corresponding amides were administered to rabbits. Since the enzyme appeared to catalyse a simple reaction and it was active towards many readily accessible substrates, the kinetics of its action seemed worthy of study. In this paper an account is given of the hydrolysis of *n*-hexanamide, which can be regarded as a typical aliphatic amide.

EXPERIMENTAL

Materials. *n*-Hexanamide was purchased (British Drug Houses Ltd.) and was recrystallized once from water before use, m.p. 101°. The *ω*-phenyl derivatives of propionic, *n*-butyric, *n*-valeric and *iso*hexanoic acids were prepared as previously described (Bray *et al.* 1950). Other carboxylic acids were purchased. Melting points of all the acids agreed with those stated in the literature.

Estimation of ammonia. The ammonia liberated by the action of the enzyme was estimated by microdiffusion (cf. Conway, 1939). The sample for analysis (1 ml.) was placed in the outer chamber of a Conway unit which contained saturated aqueous KBO_2 (1 ml.). The inner chamber contained 1 ml. of a solution of boric acid (0·5% in 20% (v/v) ethanol) which also contained bromocresol green (0·0033%) and methyl red (0·0066%). The unit was sealed with tragacanth gum adhesive (Conway & O'Malley, 1942) and left at ordinary temperature for 2 hr. The contents of the centre chamber were then titrated to a pink colour with 0·01 N-H_2SO_4. In control experiments recoveries of known amounts of ammonia ranged from 91·4 to 101·0%, the average recovery in seventeen experiments being 95·5%. *n*-Hexanamide was not significantly hydrolysed by KBO_2 under these conditions and the presence of the compounds used as inhibitors was without effect.

Preparation of digests. Rabbit-liver extract (1 ml.), prepared as described previously (Bray *et al.* 1948), was added to substrate contained in 5 ml. of buffer of appropriate pH. The buffer was a solution 0·06 M with respect to KH_2PO_4, H_2BO_3, HCl and acetic acid, and was adjusted to the required pH by addition of 0·3 N-NaOH (cf. Britton, 1942). Carboxylic acids used as inhibitors were adjusted to the appropriate pH by addition of NaOH.

The conditions were essentially as used previously, but two points require further comment. Bray *et al.* (1948) obtained preliminary evidence that dialysis had no effect upon the activity of the enzyme in liver extracts. These experiments have now been confirmed and extended (unpublished results). This suggests that no diffusible cofactor is involved in the action of the enzyme and, from a practical standpoint, implies that the effect of dilution is straightforward. It was also observed that the enzyme had little activity in salt-free solution. This has not been investigated further, but is of considerable theoretical interest. In this connexion it may be recalled that Bray *et al.* (1949*a*) showed that the substitution of a veronal for a phosphate buffer considerably reduced the activity of the enzyme towards *p*-nitrobenzamide.

Plan of experiments. Before mixing, the components of the digests were warmed to the temperature of the thermostat bath (37·2±0·2° unless stated otherwise) and the time of mixing was taken as zero time for the experiment. Samples (1 ml.) were withdrawn at intervals and transferred to Conway units for estimation of ammonia. The

appropriate controls, e.g. incubation of extract without substrate and substrate without extract, were carried out simultaneously. Where long incubation periods were involved, the observed results were corrected for loss of enzymic activity. The corrections were based on control experiments in which liver extract with buffer was maintained at the incubation temperature; mean hydrolysis rates were determined and initial rates calculated at intervals throughout the course of the main experiment. The results provided an activity decay curve from which correction factors for any given time could be derived. There was no significant spontaneous hydrolysis of *n*-hexanamide in 20 min. at pH 8·7 and 37·2°. Above pH 8·8 slight spontaneous hydrolysis was observed, the maximum extent being 0·2% at pH 10·6, the highest pH used.

Calculation of results

Calculation of initial rates. Ideally, initial rates should be measured over a very short period at the beginning of a reaction, but this is not often practicable. The difficulty is commonly overcome by extrapolation to zero time of rates determined over longer reaction periods. In the present investigation a direct use of extrapolation was not practicable, since the necessary controls and duplicates entailed about twenty analyses for the determination of each point on a curve. Instead, the extent of reaction was determined after a certain time and the reaction constants (V, K_S, K_I, etc.) were derived from the mean velocity of reaction and the calculated substrate concentration, s_x, corresponding to this by the procedure normally used for instantaneous rates and substrate concentrations. Fisher & Parsons (1953) have applied an essentially similar, but less extensive, treatment to the study of the intestinal absorption of glucose. Walker & Schmidt (1945) and Sweat, Samuels & Lumry (1950) have applied different mathematical treatment to experimental results obtained for comparatively long incubation periods. The function s_x is defined as that substrate concentration which corresponds to a mean velocity, $v_{mean}=(s_0-s_t)/t$, where s_0 is the initial substrate concentration and t_t that at time t, assuming the Michaelis–Menten relation to hold for the reaction. The advantage of the use of such a substrate concentration is that it refers to longer periods of time than do initial rates. The Michaelis–Menten relation may be written

$$v=-\frac{ds}{dt}=\frac{V}{K_S/s+1}, \qquad (1)$$

where s is the substrate concentration, K_S the Michaelis constant, v the velocity of the reaction, V the theoretical limiting velocity of the reaction, i.e. in the absence of inhibition by substrate. Also

$$v_{mean}=\frac{s_0-s_t}{t}=\frac{V}{K_S/s_x+1}. \qquad (2)$$

Rearranging eqn. 2

$$s_x=\frac{(s_0-s_t)\,K_S}{Vt-(s_0-s_t)}. \qquad (3)$$

The term Vt may be evaluated by rearranging eqn. 1 and integrating between the limits 0 and t, whence

$$Vt=K_S \ln (s_0/s_t)+s_0-s_t. \qquad (4)$$

Substituting from eqn. 4 into eqn. 3

$$s_x=\frac{s_0-s_t}{\ln (s_0/s_t)}. \qquad (5)$$

Thus, using observed mean velocities and calculated values of s_x, characteristic functions of enzymic reactions, e.g. K_S and V, may be evaluated by the same procedures as those used when initial rates are known.

It can be shown that the above treatment applies also to systems containing a constant concentration of inhibitor, as follows.

The equation for the rate v_i, of an enzymic reaction in the presence of a reversible inhibitor of concentration i is, in its general form,

$$v_i=\frac{V}{\frac{K_S}{s}\left(1+\frac{i}{K_I}+\frac{i^2}{K_IK_{II}}+\ldots\right)+\left(1+\frac{i}{K_{SI}}+\ldots\right)}, \qquad (6)$$

where K_I, K_{II} and K_{SI} are the dissociation constants for reactions 8, 9 and 10. Eqn. 6 may be written

$$v_i=\frac{V'}{K'_S/s+1}, \qquad (7)$$

where V' and K'_S are multiples of V and K_S, constant at constant inhibitor concentration. Eqn. 5 may be deduced as before.

When the enzyme is appreciably inhibited by substrate, eqn. 5 is no longer valid but, as the reaction rate then changes only slowly with changing substrate concentration, mean rates and values for s_x may be taken as identical with instantaneous rates and the corresponding substrate concentrations, provided that the extent of the reaction is not too great. In practice, eqn. 5 cannot be used if the reaction has proceeded to further than about 60% completion, since beyond this, small errors in the determination of concentrations lead to disproportionately large errors in determined values of V and K_S.

Calculation of kinetic constants. In the present study the kinetics of *n*-hexanamide hydrolysis could be accounted for on the assumption that one molecule of enzyme can combine with (*a*) one or two molecules of substrate, (*b*) one molecule of inhibitor together with one molecule of substrate, or (*c*) two molecules of inhibitor. The kinetic equations resulting from these assumptions may be derived by considering the equilibria

$$\begin{matrix} E+S\rightleftharpoons ES \\ E+I\rightleftharpoons EI \end{matrix} \qquad (8)$$

$$\begin{matrix} ES+S\rightleftharpoons ESS \\ EI+I\rightleftharpoons EII \end{matrix} \qquad (9)$$

and

$$ES+I\rightleftharpoons ESI, \qquad (10)$$

for which the dissociation constants are K_S, K_I, K_{SS}, K_{II}, K_{SI}, respectively. E, S and I represent enzyme, substrate and inhibitor molecules, while ES, EI, ESS, ESI and EII represent the appropriate complexes. The mass-action equations may be formulated:

$$e=e_SK_S/s, \qquad (11)$$

$$e_I=ei/K_I=e_SK_Si/sK_I, \qquad (12)$$

$$e_{SS}=e_Ss/K_{SS}, \qquad (13)$$

$$e_{II}=e_Ii/K_{II}=e_SK_Si^2/sK_IK_{II}, \qquad (14)$$

and

$$e_{SI}=e_Si/K_{SI}, \qquad (15)$$

where e, e_S, e_I, e_{SS}, e_{II} and e_{SI} are the concentrations of E, ES, EI, ESS, EII and ESI respectively. If the total concentration of the enzyme is e_T,

$$e_T=e+e_S+e_I+e_{II}+e_{SI}+e_{SS}, \qquad (16)$$

and the ratio of the rate v_i of the inhibited reaction to the 'maximum velocity' is given by

$$V/v_i = e_T/e_S. \quad (17)$$

Substituting from eqns. 11–15 and 17 into eqn. 16 and rearranging,

$$\frac{s}{v_i} = \frac{K_S}{V}\left(1+\frac{i}{K_I}+\frac{i^2}{K_I K_{II}}\right)+\frac{s}{V}\left(1+\frac{i}{K_{SI}}\right)+\frac{s^2}{VK_{SS}}. \quad (18)$$

For a reaction in the absence of inhibitor, eqn. 18 reduces to

$$s/v = K_S/V + s/V + s^2/VK_{SS}, \quad (19)$$

where v is the velocity of the uninhibited reaction. On account of inhibition by substrate, the observed maximum velocity ($V_{obs.}$) is smaller than V, the relation between the two being (see Haldane, 1930):

$$V = V_{obs.}\,[1+2\sqrt{(K_S/K_{SS})}]. \quad (20)$$

$V_{obs.}$ occurs at a finite substrate concentration, $s_{crit.}$, which is related to K_S and K_{SS} by $s^2_{crit.} = K_S K_{SS}$.

The constants K_S, K_I, K_{SS} and K_{SI} were evaluated graphically by plotting values of s/v against s, the intercept on the s/v axis being K_S/V and the slope $1/V + 2s/VK_{SS}$ (by differentiation of eqn. 19). The slopes of tangents to the curve were determined and plotted against s, the plot being a straight line of slope $2/VK_{SS}$ and intercept $1/V$. Hence both K_S and K_{SS} could be determined. For a reaction in the presence of an inhibitor other than substrate, the slope becomes

$$(1+i/K_{SI})/V,$$

and a plot of this slope against i gives K_{SI}; the intercept of the plot of s/v_i and s is

$$(1+i/K_I+i^2/K_I K_{II})\,K_S/V,$$

and the plot of this intercept and i is a curve having an intercept of K_S/V and an initial slope K_S/VK_I. A further plot of tangents to this curve and i has a slope of $2K_S/VK_I K_{II}$. In this way K_I and K_{II} can be evaluated.

Mean velocities and values for s_x have been used throughout this paper.

RESULTS

Effect of variation of pH. The results of a preliminary investigation are shown in Fig. 1. These were obtained by determining the percentage hydrolysis in 20 min. of a substrate solution of fixed concentration (5 or 20 mM). The experimental values obtained were corrected for enzyme decay (p. 168) and the spontaneous hydrolysis of substrate. The curve in Fig. 1 shows the pH/activity curve for the pH range in which the variation of rate is reversible. Above pH 9·2 the enzyme is irreversibly inactivated at 37·2°. It seems likely that, correcting for irreversible inactivation due to pH, the activity curve of the enzyme forms a plateau from about pH 8·7 upwards. This pH was designated optimum pH since it was the most alkaline value at which irreversible inactivation is negligible. It can be seen from Fig. 1 that the same curve fits the experimental values obtained with substrate concentrations of either 5 or 20 mM. This suggests that the value of the Michaelis constant does not vary greatly with pH, and this interpretation is supported by the two values for this constant at pH's 7·3 and 8·7 shown in Table 1.

Effect of changes in substrate concentration. Experiments were performed in which the mean rates of hydrolysis of *n*-hexanamide in solutions of various initial concentrations were determined. The results are shown in Fig. 2. It can be seen that there is appreciable inhibition by substrate when the value of s_x exceeds 13 mM. Values for the apparent dissociation constants K_S, K_{SS} and $s_{crit.}$ deduced as described above, are given in Table 1.

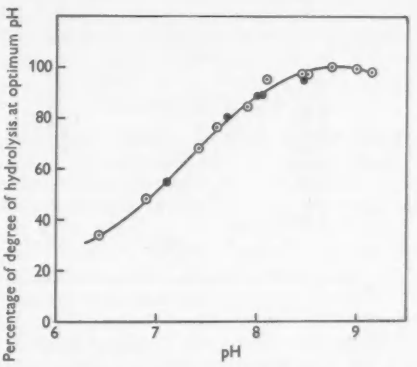

Fig. 1. pH/activity curve for rabbit-liver amidase at 37·2°. Substrate *n*-hexanamide: ⊙, 5 mM; ●, 20 mM. Amidase and buffer prepared as described in the text. Percentage hydrolysis was determined after incubation for 20 min. and is corrected for enzyme decay and spontaneous hydrolysis.

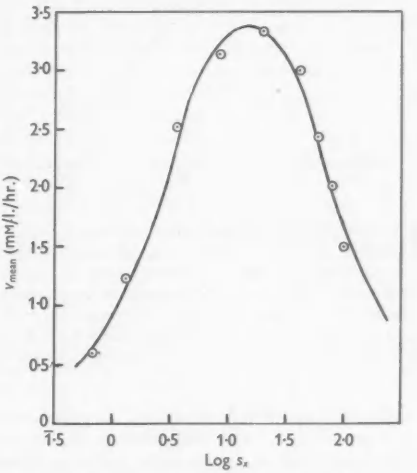

Fig. 2. Substrate concentration (mM)/velocity curve for hydrolysis of *n*-hexanamide by rabbit amidase at 37·2° and pH 8·7. Amidase and buffer as described in text. Incubation for 20 min.

Table 1. *Michaelis and inhibitor dissociation constants for rabbit-liver amidase with varying concentrations of* n*-hexanamide and added* n*-hexanoate*

Constants and concentrations of substrate (*n*-hexanamide), *s*, and of inhibitor (*n*-hexanoate), *i*, expressed as mM. All determinations at 37·2° for 20 min.

Concentration range of						
s	*i*	K_S	K_{SS}	K_I	K_{SI}	$s_{crit.}$
			pH 7·3			
2–83	0	3·9	170	—	—	26
2–83	0	4·2	182	—	—	28
2–83	0	3·2	234	—	—	27
	Mean	**3·8**	**195**	—	—	**27**
			pH 8·7			
5–83	0	7·3	31	—	—	15
5–83	0	5·3	39	—	—	14
5–83	0	6·3	40	—	—	16
2–80	0–100	8·0	32	—	42	19
2–10	0–500	—	—	12	76	—
2–10	0–500	3·4	—	7	—	—
3–7	0–100	—	—	9	84	—
1–100	0	5·1	42	—	—	—
	Mean	**5·9**	**37**	**9**	**67**	**16**

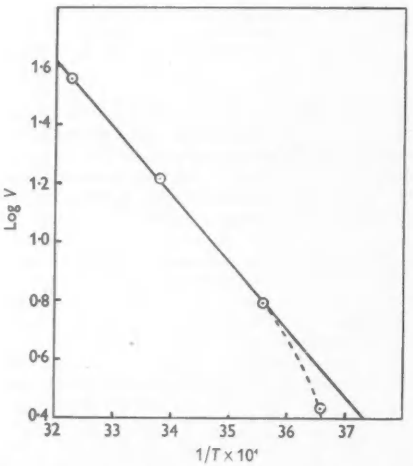

Fig. 3. Temperature/theoretical limiting velocity curve for hydrolysis of *n*-hexanamide by rabbit-liver amidase at the optimum pH. Each point represents the mean of three values of *V*, each determined in an experiment similar to that shown in Fig. 2. Experiments at 37·2, 23·1, 8·1 and 0·3° were carried out at pH 8·7, 9·0, 9·2 and 9·7 respectively. Corresponding incubation times were 20, 30, 60 and 120 min.

Effect of changes in temperature. Experiments of the type referred to in the previous section were carried out over a temperature range and using eqn. 20. Each experiment consisted of a simultaneous determination of $V_{obs.}$, K_S and K_{SS}, and hence *V*, at two temperatures. The values obtained were normalized by use of the 'incomplete block' method (Yates, 1936–7). From the values obtained in this way the apparent energy of activation for the conversion of enzyme-substrate complex into free enzyme and product was evaluated, being numerically equal to the slope of a plot of $1/T$ and log *V*, as in Fig. 3. It was 10·5 kcal. (S.D. 0·08) over a range 8·1–37·2°, but there was a deviation below 8·1°, the cause of which has not been determined. The optimum pH of enzyme activity varied with temperature being about 9·7 at 0·3°. Cognizance was taken of this in the preparation of digests.

Effect of hexanoate ion. Progress curves, even after correction for inhibition by *n*-hexanamide and for enzyme decay, did not follow the course expected from the application of theoretical equations. For example, the degree of hydrolysis of 90 mM and 72 mM *n*-hexanamide solution remained stationary at 70 and 91% respectively, even on prolonged incubation. This suggested that reaction products might be inhibitors of the hydrolytic process and it was shown that the inhibitor was the hexanoate ion and not ammonia. Fig. 4 shows the relation between mean velocity and s_x in the presence of various concentrations of added hexanoate (cf. Lineweaver & Burk, 1947). It can be interpreted as showing that the constants K_I, K_{II} and K_{SI} for the reaction have significant values. Those for K_I and K_{SI} are included in Table 1. Values for K_{II} can only be determined under conditions which result in little enzymic activity, and consequently lack precision. Calculation from such values gives values of K_{II} in the range 200–1000 mM.

Inhibition by hexanoate ion formed as a reaction product is appreciable only when a relatively large amount of amide (corresponding, for example, to about 50% hydrolysis of a 50 mM solution of amide)

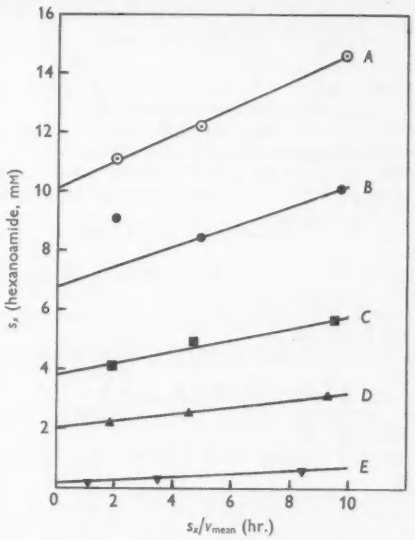

Fig. 4. The relation between s_x and mean velocity for hydrolysis of *n*-hexanamide by rabbit-liver amidase at pH 8·7 and 37·2° in the presence of varying amounts of added *n*-hexanoic acid. Other conditions as for Fig. 2. Concentration of hexanoate (mM) in *A*, 500; *B*, 375; *C*, 250; *D*, 125 and *E*, 0.

Table 2. *Overall concentrations of carboxylic acids causing* 50 % *inhibition of the hydrolysis of* n-*hexanamide by rabbit-liver amidase*

n-Hexanamide (30 mM) was incubated with amidase in buffer, pH 8·7, at 37·2° in each case. Very feeble inhibition is indicated by L and no inhibition by ∞.

Unsubstituted and ω-phenyl-substituted aliphatic acids		Substituted benzoic acids	
Acid	Concn. (mM)	Substituent	Concn. (mM)
Formic	∞	None	178
Acetic	∞	*m*-Amino	∞
Propionic	∞	*p*-Amino	∞
Butyric	∞	*m*-Hydroxy	∞
Valeric	L	*p*-Hydroxy	L
Hexanoic	22	*p*-Carboxy	∞
Heptanoic	15	*m*-Nitro	106
*iso*Hexanoic	∞	*p*-Nitro	77
*cyclo*Hexanecarboxylic	120	*m*-Methyl	L
		p-Methyl	36
Phenylformic (benzoic)	178	*p*-Methoxy	L
Phenylacetic	114	*p*-Cyano	150
Phenylpropionic	85	*p*-Fluoro	70
Phenylbutyric	100	*m*-Chloro	50
Phenylvaleric	21	*p*-Chloro	26
p-Aminophenylacetic	150	*p*-Bromo	22
p-Nitrophenylacetic	200	*p*-Iodo	6
Phenylacrylic	44	—	—

has been hydrolysed and so does not affect determinations of initial rates or those of mean rates made over a short period at the beginning of a reaction.

The effect of certain other carboxylate ions. As described in the previous section, the hexanoate ion inhibits the hydrolysis of *n*-hexanamide by rabbit-liver amidase. The results obtained with some other carboxylate ions are summarized in Table 2, which shows the concentrations (mM) necessary for 50 % inhibition of hexanamide hydrolysis. There appears to be no obvious correlation between the structure and activity of these inhibitors.

DISCUSSION

The reaction system involved in the enzymic hydrolysis of *n*-hexanamide is complex, since the process is inhibited by substrate and by one of the hydrolysis products; inhibition by the hexanoate ion is neither strictly competitive nor non-competitive. Values for five dissociation constants have been obtained. There seems to be no reason why such an extensive analysis should not be possible for other enzymes.

The chief finding of the present investigation is that there are two groups on the catalytic site of the amidase molecule which can combine with substrate or inhibitor, since values were obtained for K_{SS}, K_{SI} and K_{II}. The affinities of the two groups for *n*-hexanamide are approximately equal at pH 8·7. If they were the same, according to the theory of step reactions (Michaelis, 1949) K_{SS}/K_S should be 4; it is in fact 6·3. Since K_I is of the same order as K_S, one group at least has about the same affinity for *n*-hexanamide and for *n*-hexanoate, but since K_{II} is very much greater than K_{SS}, the second group has presumably greater affinity for *n*-hexanamide. Another observation which has a bearing on the problem of relative affinities is that K_{SS} shows a greater degree of pH dependence than does K_S. This can be accounted for by suggesting that the two groups differ in their electrical behaviour. An ionic group would be expected to change in affinity with pH. The variation in the value of $s_{crit.}$ with pH (Table 1) is accounted for almost entirely by the variation in the value of K_{SS}.

The present study provides a very satisfactory example of substrate inhibition since almost complete symmetry of the $(\log s)/v_{mean}$ graph was found for a wide range of substrate concentrations.

SUMMARY

1. A study has been made of the hydrolysis of *n*-hexanamide by rabbit-liver amidase.

2. The enzyme is inhibited by substrate and by *n*-hexanoate ion. The inhibition by the latter is not strictly competitive or non-competitive.

3. Values for four apparent dissociation constants have been determined and the magnitude of another calculated.

4. Preliminary experiments have been performed on the inhibition of *n*-hexanamide hydrolysis by carboxylate ions other than *n*-hexanoate.

5. The effect of temperature on the hydrolysis has been studied: the apparent energy of activation is 10·5 kcal. between 8·1 and 37·2°.

6. The optimum pH of the reaction is about 8·7 at 37·2°: at higher pH values irreversible inactivation takes place.

7. The results are discussed in terms of the relative affinities of two groups on the catalytic site of the enzyme for substrate/or inhibitor.

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Interference by Amino Acids in the Estimation of Sugars by Reductometric Methods

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It is well known that amino acids interfere with the determination of reducing sugars by alkaline copper and ferricyanide reagents. The interference is negligible in blood-sugar analysis owing to the low amino acid content of blood, but in such materials as protein hydrolysates or bacteriological culture media the interference may be very large. It was indeed the unreliability of reductometric methods for the estimation of carbohydrate groups in proteins that led Sørensen & Haugaard (1933) to apply the orcinol method to this problem, and similar techniques have since been developed using carbazole (Gurin & Hood, 1939), anthrone (Morris, 1948), etc. While these colorimetric methods are more specific than reductometric methods, they are less precise, and the concentrated sulphuric acid employed in most of them produces charring with amino acids such as tryptophan. Many analysts would prefer to use reductometric methods if means could be found for overcoming amino acid interference, and this has been the object of the present investigation.

As a preliminary, the effects of pure amino acids and protein hydrolysates on alkaline copper and ferricyanide reagents were investigated. Some observations along these lines have been reported by Holden (1926), Hewitt (1938) and Munday & Seibert (1933). Marked differences were found in the reducing effects of different amino acids and between different sugar reagents.

To remove interfering amino acids, a number of the deproteinizing reagents used in blood-sugar analysis was tried, since many of them are claimed to remove other interfering substances. None of them was found effective, however, when applied to materials such as protein hydrolysates.

Synthetic ion-exchange resins have been used by a number of workers to remove various interfering substances prior to sugar analysis. Serbiá (1947) used them to remove breakdown products of sugars ('glutose') which had reducing power and were produced by heat treatment of molasses. Partridge & Westall (1948) treated extracts of biological materials with resins as a preliminary to the chro-

matographic analysis of sugars. Williams, Potter, Bevenue & Scurzi (1949) used resins for clarifying extracts of potatoes before reducing-sugar analysis, and Williams, Bevenue & Washauer (1950) extended this work with a comprehensive, comparative study of the use of various resins in the analysis of fresh and dehydrated vegetables.

We describe the use of ion-exchange resins in the present paper, and it is hoped that the results obtained in estimating sugar in the presence of relatively large amounts of amino acids will be of use to other workers.

MATERIALS AMD METHODS

Sugars. 'AnalaR' D-glucose and Laboratory Reagent Grade galactose, fructose, mannose, ribose from British Drug Houses Ltd., dried over P_2O_5.

Amino acids. B.D.H. Laboratory Reagent Grade.

Casein hydrolysate. 'Difco Bacto Casamino Acids' supplied by Baird and Tatlock (London), Ltd. This is a purified acid hydrolysate and the powdered material was used as supplied.

Synthetic resins. IR-120 (H), IR-4B (OH) and IRA-400 (OH) from B.D.H. Ltd. were used as supplied after air drying. Zeo-Karb 225 from the Permutit Co. Ltd. was converted into the H form by treatment with 10% HCl and thorough washing. Details of these resins and regeneration procedures are given in 'Notes on the Laboratory Use of Ion-Exchange Resins' from B.D.H. Ltd.

Reductometric methods. For details of the techniques the original papers should be consulted. (*a*) The copper method of Somogyi (1945), as described for the estimation of up to 3 mg. of glucose in the sample, 0·01 N-$Na_2S_2O_3$ was used; (*b*) the ferricyanide method of Fujita & Iwatake (1931)—(F & I), modified by Herbert (unpublished), for the estimation of up to 2·5 mg. of glucose in the sample, 0·05 N-$Na_2S_2O_3$ was used; (*c*) the ferricyanide method of Hagedorn & Jensen (1923)—(H & J) as described for the estimation of up to about 0·36 mg. of glucose in the sample, 0·005 N-$Na_2S_2O_3$ was used. The reagents described for deproteinization in each method were used only as stated in the text.

EXPERIMENTAL AND RESULTS

Reduction by solutions of pure amino acids. Nineteen amino acids were examined in eleven groups, similar ones such as glycine, alanine, valine, leucine and isoleucine being included together. Tyrosine, cystine and tryptophan were dissolved in a small volume of HCl, diluted to the required concentration, and samples were adjusted to near neutrality with NaOH before testing. Appreciable reduction was obtained as follows: of all three reagents by cystine, tryptophan and tyrosine; of F & I reagent by proline and hydroxyproline; of H & J reagent by methionine. The following amino acids gave little or no reduction of copper and ferricyanide: glycine, alanine, valine, leucine, isoleucine, arginine, lysine, histidine, serine, threonine, glutamic acid, aspartic acid and phenylalanine.

In general the amount of reduction of copper was less than that of ferricyanide and was usually not proportional to the amount of amino acids present, e.g. the reducing values as glucose for 2 and 4 mg. of DL-tryptophan were 0·41 and 0·49 mg. A more quantitative effect was obtained with L-cystine, 1, 3 and 5 mg. giving reducing values equivalent to 0·65, 1·6 and 2·2 mg. as glucose respectively.

Reducing power of casein hydrolysate. Casein contains little cystine (0·4%), tryptophan is destroyed by acid hydrolysis and the hydrolysate did not significantly reduce alkaline copper; 12·5, 18·75 and 25 mg. gave reducing values with the Somogyi reagent equivalent to 0·08, 0·1 and 0·1 mg. glucose respectively. With the F & I reagent, these amounts of casein hydrolysate gave values of 0·76, 1·16 and 1·6 mg. as glucose respectively.

The reducing powers of mixtures of amino acids and glucose. The reducing powers of samples containing from 5 to 50 mg. of casein hydrolysate and 2 mg. of glucose together with similar solutions made just alkaline to phenolphthalein were determined by the Somogyi method. Neutralized samples were included in the test in case the buffering power of the larger amounts of amino acids was affecting the results. Results are shown in Table 1, and it may be seen that either low or high recoveries of glucose were obtained according to the amount of casein hydrolysate present. Mixtures of tyrosine and glucose gave less reduction than the same quantity of glucose alone, e.g. 2 mg. of glucose with 0·25, 0·5 and 1·0 mg. of tyrosine gave recoveries of 1·58, 1·85 and 1·87 mg. of glucose. It is evident that interference by amino acids in this method is not necessarily explained by their reducing power, and in fact inhibition of reduction by glucose occurs in certain cases. This complicated interference in the presence of glucose was found to be very dependent on the time the sample and the copper solution were left in the boiling-water bath. Whereas glucose alone gave maximum reduction at or just after 8 min. in the bath, the maximum reducing power of mixtures of glucose and amino acids was not reached until about 12 min. Casein hydrolysate (12·5 mg.) with 2 mg. of glucose gave reducing power values after 8, 10 and 12 min. equivalent to 2·04, 2·26 and 2·28 mg. of glucose respectively. Samples containing 25 mg. of casein hydrolysate and 0·5, 1·0 and 2 mg. of glucose were estimated by the F & I method with recoveries of 1·85, 2·09 and 2·5 mg. glucose respectively.

Treatment with deproteinizing reagents. As the F & I reagent was the one most affected by amino acids, it was used to investigate the effect of deproteinizing reagents. Both $Cd(OH)_2$ (Fujita & Iwatake, 1931) and $BaSO_4$–$Zn(OH)_2$ (Somogyi, 1945) removed cystine completely, probably because of the insolubility of this acid under such conditions and barium–zinc precipitation decreased the reducing power of tryptophan.

Table 1. *Reduction of Somogyi's (1945) copper reagent by mixtures of glucose and casein hydrolysate*

All solutions contained 2 mg. of glucose.

Casein hydrolysate (mg.)	Recovery as glucose (mg.)	
	No treatment	Neutralized (alkaline to phenolphthalein)
5	1·95	1·98
10	2·20	2·28
20	2·24	2·24
30	2·18	2·19
40	1·88	2·08
50	1·61	1·89

The reducing powers of solutions of casein hydrolysate before and after treatment with $Cd(OH)_2$ were practically identical. Treatment of similar solutions with $BaSO_4$–$Zn(OH)_2$ or by the $HgSO_4$ method of West, Scharles & Peterson (1929) lowered their reducing powers only slightly.

Treatment with cation-exchange resins. IR-120 (H) and Zeo-Karb 225 (H) are synthetic cation-exchange resins which exchange hydrogen ions for other positively charged ions. To test their efficiency the following experiment was made: 50 mg. of casein hydrolysate was dissolved in 10 ml. of water and about 1 g. of resin was added. The mixture was shaken on a rotary shaker for 15 min. and after the resin had settled, a sample (100 μl.) was applied to paper in parallel with an untreated sample (20 μl.) for examination by paper chromatography (Consden, Gordon & Martin, 1944). After development and spraying, the resin-treated sample showed only the merest trace of ninhydrin-reacting material, in contrast to the untreated sample, which showed a large amount. Neither of these resins removed glucose from solution, but in the presence of large quantities of cations the acid produced was often sufficient to interfere with the subsequent estimation and had to be neutralized. Acid formation could be avoided by including an anion-exchange resin; IR-4B (OH) was found satisfactory, whereas IRA-400 (OH) removed nearly all the glucose present.

Table 2. *Shaking times with IR-120 (H) and Zeo-Karb 225 (H) for the removal of amino acids*

Ferricyanide method (Fujita & Iwatake) used. Details are given in the text.

	Reducing power of 25 mg. of casein hydrolysate expressed as glucose (mg.)			
Time of shaking (min.) ...	1	2	3	15
Zeo-Karb 225 (H)	0·34	0·19	—	0·103
IR-120 (H)	0·13	0·12	0·12	0·103
No treatment		1·6		

Table 3. *Reduction of Somogyi's (1945) copper reagent by mixtures of various sugars and casein hydrolysate after treatment with IR-120 (H) and IR-4B (OH)*

Details are given in the text.

	Recovery as glucose (mg.) in duplicate tests	
Sugar	Alone, no treatment	+25 mg. casein hydrolysate with resin treatment
Glucose	1·0, 1·0	1·0, 1·0
Galactose	0·815, 0·815	0·815, 0·815
Fructose	0·91, 0·90	0·90, 0·90
Mannose	0·91, 0·93	0·93, 0·93
Ribose	0·67, 0·68	0·66, 0·66

Table 4. *Reduction of Somogyi's (1945) copper solution by barium–zinc-treated solutions of mixtures of glucose, casein hydrolysate and bovine albumen before and after treatment with IR-120 (H) and IR-4B (OH)*

Each sample contained 10 mg. of bovine albumen and 25 mg. of casein hydrolysate. Other details are in the text.

	Recovery as glucose (mg.) in duplicate tests	
Glucose (mg.)	No treatment	Resin treated
0·5	0·66, 0·65	0·51, 0·51
1·0	1·16, 1·19	0·99, 0·99
1·5	1·69, 1·71	1·49, 1·48
2·0	2·14, 2·14	1·98, 1·98

To find the optimum time for treatment with IR-120 (H) and Zeo-Karb 225 (H), samples containing 50 mg. of casein hydrolysate in 10 ml. of water were shaken with approximately 1 g. of resin for various times and after filtering through Whatman no. 42 paper, a 5 ml. portion ($\equiv$25 mg. casein hydrolysate) was tested for reducing power by the F & I method. The results (Table 2) showed that complete removal of interference was not obtained but the residual effect after 15 min. was not more than 7% of that before treatment and this time was adopted.

Solutions (10 ml. each) containing 50 mg. of casein hydrolysate and 2 mg. of glucose were shaken with approx. 1 g. each of IR-120 (H) and IR-4B (OH) for 15 min. After filtration through Whatman no. 42 paper, 5 ml. samples gave the correct reducing values with the Somogyi reagent. Galactose, fructose, mannose and ribose in the presence of casein hydrolysate also gave correct recoveries after resin treatment (Table 3).

Solutions (10 ml. each) containing 50 mg. casein hydrolysate and 1·0, 2·0 and 4·0 mg. of glucose were treated with IR-120 (H) and after filtration 5 ml. samples gave reducing-power values equivalent to 1·14, 2·13 and 4·2 mg. glucose respectively with the F & I reagent.

Treatment of samples containing protein and amino acids. Duplicate portions (6 ml.) of solutions containing 50 mg. of casein hydrolysate, 20 mg. bovine albumen and 1, 2, 3 and 4 mg. of glucose were treated with 2 ml. 5% $ZnSO_4$, $7H_2O$ and 2 ml. 0·3 N-$Ba(OH)_2$. After mixing and centrifuging, the clear supernatants from one series were shaken with 1 g. each of IR-120 (H) and IR-4B (OH) for 15 min. After filtration through Whatman no. 42 paper, 5 ml. samples were tested for reducing power with the Somogyi reagent in parallel with 5 ml. samples of the non-resin-treated supernatants. Correct recoveries were obtained from resin-treated samples (Table 4).

Estimation of similar solutions by the F & I method using the $Cd(OH)_2$ deproteinizing reagent and IR-120 (H) followed by neutralization of the liberated acid with sodium carbonate gave glucose recoveries close to the theoretical.

DISCUSSION

The results of the present work indicate that sugar cannot be accurately estimated in the presence of relatively large amounts of amino acids by the Somogyi (1945), the Fujita & Iwatake (1931) or the Hagedorn & Jensen (1923) methods, although the copper method is superior for this purpose. Interference in the copper method cannot be explained by the reducing power of amino acids, since certain of these inhibit the reduction by sugar, e.g. tyrosine and glucose gave less reduction than the same quantity of sugar alone. Recovery of glucose from

mixtures of casein hydrolysate and glucose depended on the relative concentrations of each. Amino acids form complexes with alkaline solutions of copper which are easily observed by their strong blue colour and they also form compounds with sugar in alkaline solution. These three factors, i.e. reducing power, copper-complex formation and sugar–amino acid compounds, probably all contribute to the interference found.

The use of ion-exchange resins extends the use of reductometric methods for the estimation of sugars and the Somogyi (1945) method will give correct recoveries with small amounts of sugar and large amounts of amino acids.

SUMMARY

1. Interference by amino acids in the reductometric estimation of glucose was investigated using copper and ferricyanide methods.

2. Copper was appreciably reduced by cystine, tyrosine and tryptophan, but in general was less affected by amino acids than was ferricyanide.

3. Although casein hydrolysate alone had little effect on copper, recoveries of glucose from samples containing it and glucose were incorrect.

4. Precipitation by barium, cadmium or mercury did not substantially lower the reducing power of a solution of acid-hydrolysed casein using the Fujita & Iwatake method.

5. A method using synthetic cation-exchange resins is described which allows the accurate estimation of glucose in the presence of a large excess of amino acids.

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